INDUCTION OF APOPTOSIS IN THE PRESENCE OF NATURAL POLYPHENOLS AND

EXPRESSION PROFILE OF K-Ras AND Akt1 IN BREAST CANCER CELL LINE MDA-MB-231

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SUBMITTED BY

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CERTIFICATE

This is to certify that the thesis entitled ***"Induction of Apoptosis in the presence of Natural Polyphenols and expression profile of K-Ras and Akt1 in Breast Cancer Cell line MDA-MB-231"*** which is being submitted by Miss Anita Singh, Roll No.412LS2060 for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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DECLARATION

I hereby declare that this project report on, **"INDUCTION OF APOPTOSIS IN THE PRESECNCE OF NATURAL POLYPHENOLS AND EXPRESSION PROFILE OF K-RAS AND AKT1 IN BREAST CANCER CELL LINE MDA-MB-231"**, is the result of the work carried out by me.Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions. The work was done under the guidance of ***Dr. Samir Kumar Patra,*** Associate Professor and Head, Department of Life Science, National Institute of Technology, Rourkela.

Date: 10th May 2014

Place: Rourkela **(Anita Singh)**

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ABSTRACT

One of the vital process by which cellular homeostasis is maintained is Apoptosis. In normal cells this program is well balanced. But any alteration in genetic and epigenetic process leads to loss of key properties like proliferation, differentiation, invasion, adhesion, and hence opened thegateway for complex set of disease known as cancer. Moreover, RAS/MEK/ERK and PI3K/AKT signaling cascade is the most frequently mutated pathway in human cancer. Oncogenic mutations in these cascade leads to deregulation of several effecter pathways that control cell proliferation, survival, apoptosis as well migration, and thus promote malignant transformation. As all natural polyphenols have anti- cancer property to some extent, we aimed at investigating the effect of natural polyphenols like curcumin and EGCG in combination with other conventional chemotherapeutic agents which are used a lot in epigenetic targeted therapies, potentially working synergistically in increasing the therapeutic effect of drugs in combat against cancer. So the present study aims at deciphering the role of curcumin and EGCG on the induction of apoptosis, inhibition of cell proliferation and change in expression level of oncogenes K-Ras and Akt1 in breast cancer cell lines. A brief study can help to pile valuable information for developing better therapeutic interventions against cancer.

Key words: apoptosis, cancer, natural polyphenols, curcumin, EGCG, K-Ras, Akt1

INTRODUCTION

Body is made up of trillion of living cells which grows, divides and dies in regular fashion to maintain cellular homeostasis. Aberration in any of the vital process due to diseased condition leads to the loss of key properties like proliferation, differentiation, invasion,adhesion etc. resulting in rise of a complex set of disease known as cancer. Various factors are responsible for onset of tumorogeneis like deletion, mutation, chromosome rearrangements etc. These alterations are broadly classified under two heads, one is genetic and the other is epigenetic mechanism.In normal cells homeostasis is achieved by maintaining a proper balance between cell death and proliferation but in cancer cell there is evasion of cell death which enhances the survivability of these cells as compared to normal ones. Various signaling pathways act in cohort to orchestrate the evasion of cell death in cancer cells like PI3/AKT and RAS/MEK/ERK pathways. Genes of PI3K/AKT and RAS/MEK/ERK pathway are mutated in such a way that they remain in permanently turn "on" state leading to the survival of cells when it is no longer needed and thus inhibiting apoptosis. This causes cancer in combination with other lethal mutations. Besides these, epigenetic alterations like DNA methylation at promoter region or post translational histone modification may lead to either suppression of tumor suppressor genes or activation of oncogenes. Thus any defect in genome, including both genetic and epigenetic alterations causes the development of cancer. It can affect almost any part of the body giving rise to myriad forms of cancer like lung cancer, breast cancer, colon cancer, prostate cancer, pancreatic cancer etc. Out of these, Breast cancer is one of the major causes of women mortality in United States than any other cancer except lung cancer. It accounts for 22.9% of all cancers in women throughout the world causing 16% of all female cancers deaths worldwide. Every year approximately 200,000 women and 2,000 men are diagnosed with breast cancer (http://www.cancer.org) so it is 100 times more common in women than in men. Day by day number of deaths caused by breast cancer in western countries is increases rapidly so it is necessary to take some appropriate and required steps to improve the treatment. With the advancement of science and technology many drugs like temoxifen, transtuzumab etc. and other effective ant- cancer therapeutics are introduced and worldwideadopted,resulting in decrease in mortality rate caused by breast cancer. At present besides these conventional chemotherapeutic agents natural polyphenols are also used a lot in epigenetic targeted therapies in human trials

potentially working synergistically in increasing the therapeutic effect of drugs in combat against cancer.

Therefore the present study aims at deciphering the role of natural polyphenols like curcumin and EGCG on the induction of apoptosis, inhibition of cell proliferation and change in gene expression of K-Ras and Akt1in breast cancer cell lines. A brief study can help to pile valuable information for developing better therapeutic intervention against cancer.

Ras is a guanosine nucleotide binding protein belonging to a class of protein named as small GTPase which involves in signal transduction within the cells. It regulates variety of biological processes that includes cell proliferation, differentiation and apoptosis. RAS and RAS related protein may also deregulated in cancers leading to increase in metastasis and invasion and decreasing apoptosis.It activates many pathways out of which MAP kinase pathway is well studied which transmit downstream signals resulting in transcription of genes involved in cell growth and cell division. It also activates AKT pathway which inhibits apoptosis.

Two forms of Ras H-Ras and K-Ras originally studied in rats during 1960s by Jennifer Harvey and Werner Kirsten respectively and named as Rat Sarcoma and then were discovered byGeoffrey M. Cooper at Harvard in human cells in 1982. Third Ras gene named N-RAS was identified in human neuroblastoma cells. The three form of human RAS i.e. H-Ras, K-Ras, N-Ras encodes a similar proteins which constitutes 188-189 amino acids.All Ras gene contains six stranded beta sheets and five alpha helices. These three human genes function as molecular switches in “on and off” states controlling intracellular network signaling. When it is bound to inactive guanosine diphosphate (GDP) it is in “off state” while in “on state” when it is bound to guanosine triphosphate (GTP). Guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) facilitate the switching between active GTP and inactive GDP. The whole mechanism is shown in fig 1.

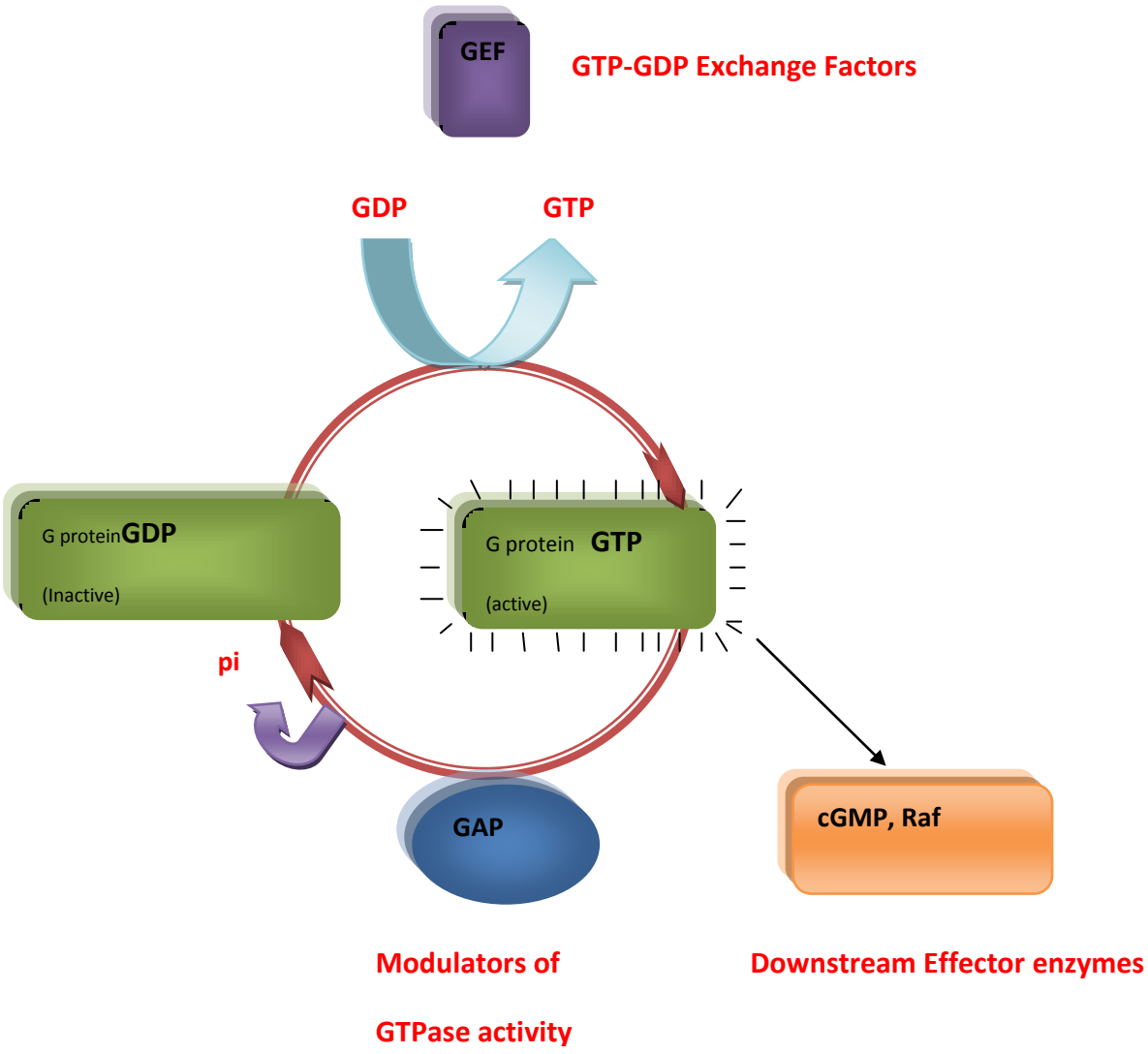


Fig1: G- protein regulation

RAS/MEK/ERK signaling pathway:

Raf serine/ threonine kinase is the best downstream effector target of RAS. When GTP binds to RAS, activates a protein kinase, Raf-1 which phosphorylates MEK on two serine residues and activates it. MEK activation phosphorylates ERK on Thr and Tyr residues and activates it. Activated EKR enters inside the nucleus and phosphorylates nuclear transcription factor Elk1 and finally activates it. This activated Elk1 joins to serum responsefactor (SRF) to stimulate the transcription and translation of a set of genes considered necessary for cell division.

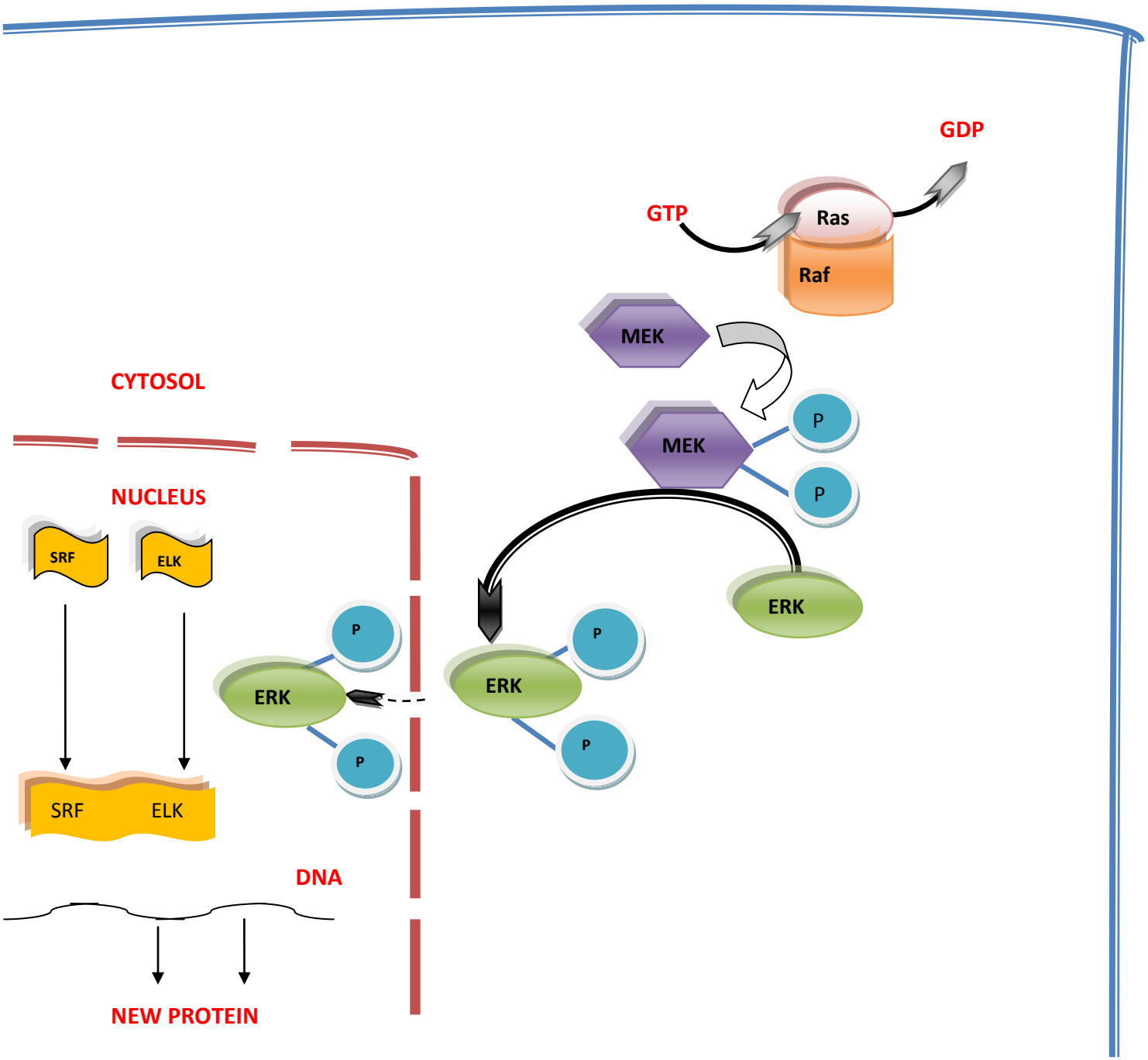


Fig2: RAS/MEK/ERK pathway

PI3K/AKT pathway:

The major component of cell membrane in eukaryotes is Phosphatidylinositol. Phosphatidylinositide kinase pohosphorylates the inositol head of phospholipid which helps in signal transduction involved in regulation of multiple cellular functions.Phosphatidylinositol 3-kinases (PI3K) having catalytic subunit p110 and regulatory subunit p85, which involves in cell growth, proliferation, differentiation, survival and motility, ultimately cause cancer. It can be activated by two pathways. In first pathway it reacts with growth factor receptors having phosphorylated tyrosine residues. As a result conformational changes occur indimmers resulting in activation of PI3. Second pathway is direct binding of PIP3 to catalytic subunit p110 via Ras.PI3Kare the second most characterized Ras effecter. It is a lipid kinaseand activated by a transmembrane signal, converting phosphatidylinositol 4, 5 bisphosphate(PIP2) to phosphotidylinositol 3,4,5trisphosphate (PIP3). This PIP3 recruits Akt and PDK1 to plasma membrane to activate downstream signaling components. When it bound toPIP3, Akt is phosphorylated and activated by phosphoinositide dependent kinase (PDK). Once Akt is activated, it can modulatenumerous substrates and regulate multiple cellular events like cell cycle progression, cell growth, cell survival, apoptosis, transcription etc by binding with downstream effectors like nuclear factor kB, BCl2 family etc. Akt could phosphorylate a pro-apoptotic protein BAD which is a member of BCl2 on its Ser136 residue, dissociating it from Bcl-2/Bcl-X complex as a result lose its pro-apoptotic function. It also activates NFkB by regulating IkB kinase (IKK) which results in the transcription of pro survival genes. Out of three isoforms of Akt i.e Akt1, Akt2, Akt3, Akt1 inhibits apoptosis and thus involved in cellular survival pathways. It has been shown that it is a critical player in oncogenesis. p85 subunit of Akt binds with the activated epidermal growth factor receptor kinase protein specially HER2 or mutational inactivation or the deletion of PTEN tumor suppressor gene results in increased kinase activity.

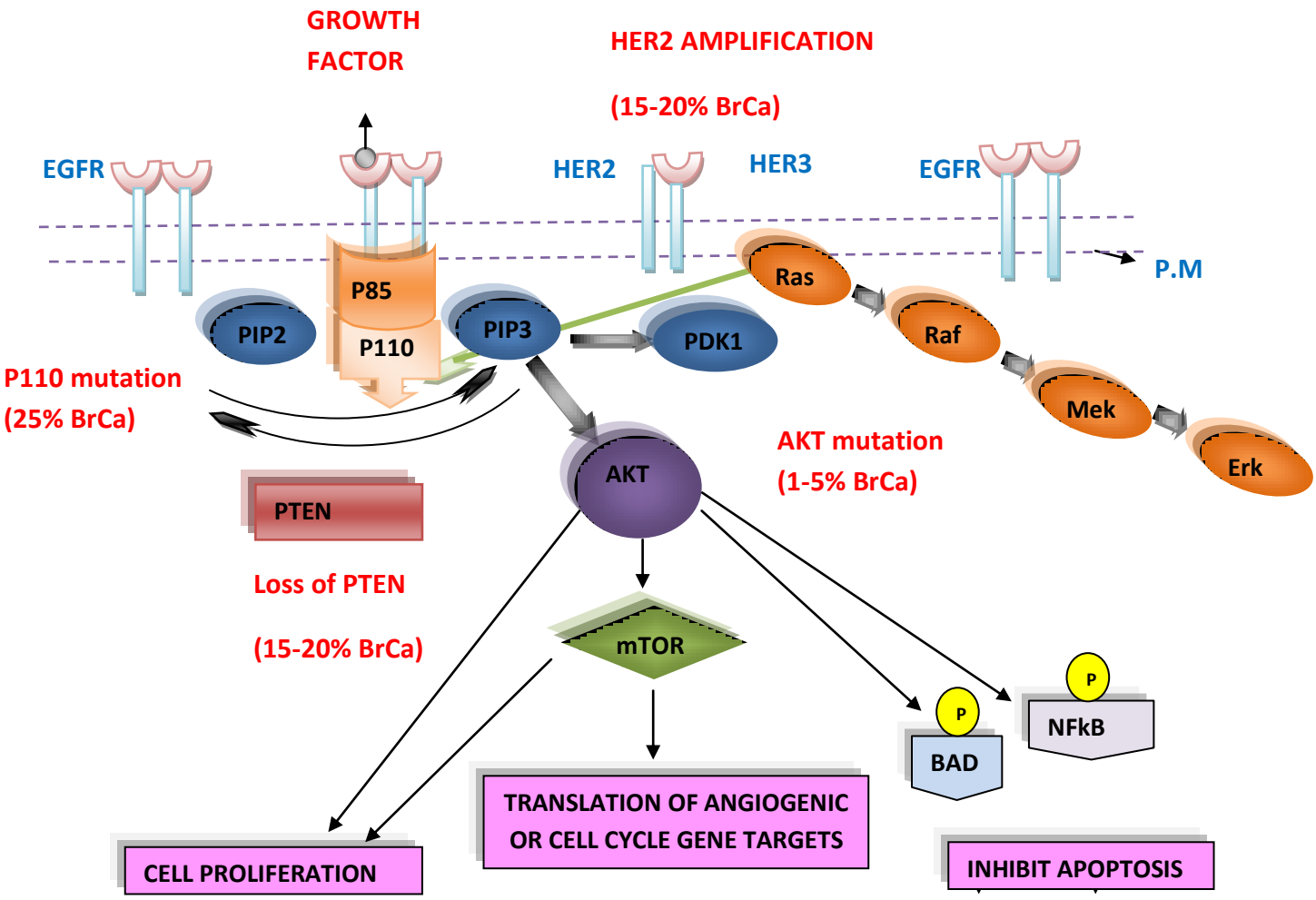


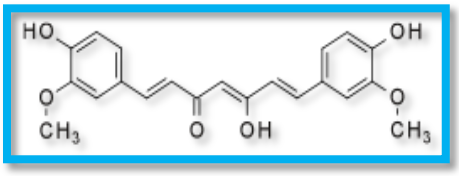
Fig3: PI3/AKT/mTOR pathway activation

EPIGENETIC MODULATORS:

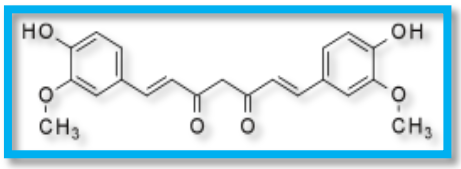
Various diseases and behavioral pathologies are emerging due to defect in gene functions and the well known example is cancer. By changing the sequence of DNA or by changing the epigenetic programming of a gene without changing the DNA sequence one can change the function of a gene. Epigenetic modulators can be used to revertsuch aberrant gene expression bytargeting DNA methylation, Histone acetylation, histone deacetylation, histone methylation, signatures. Till now only few drugs like curcumin, SAHA, SAM, TSA, EGCG etc. have shown success in clinical trials.

CURCUMIN as an epigenetic modulator:

Curcumin is a diarylheptanoid, a member of zingiberaceaefamily extracted from the rhizomes of *Curcuma longa*is a naturally occurring phytochemical receiving attention from the investigators working in field of tumorigenesis. It inhibits growth, invasion and metastasis of tumor cells, inducing apoptosis and sensitizing the tumor cells towards chemotherapeutic drugs.It is a polyphenol component also having antioxidant and anti-mutagenicproperties and exists in several tautomericforms. The enol form is energetically more stable than compared to ketoform. The general formula is 1, 7-Bis (4-hydroxy-3-methoxyphenyl) 1, 6-heptadiene-3,5dione.



Enol form



keto form

Fig4:Structure of curcumin

Curcumin could exert its biological activities even at low concentration through epigenetic modulation. It act as DNA methyltransferase inhibitor and hence known as DNA hypomethylating agent. It can block the catalytic thiolate of DNMT1 with the IC_{50} of 25 µM leading to an inhibition of DNA methylation [1]. It also renew the balance between histone acetyltransferase activity to activate and histone deacetylase(HDAC 1, 3, 4, 5, 8)activity to inactivate the expression of genes implicated in cancer death and progression. It can suppress the HDAC-i activated tumor development proteins and cell migration in vitro on combining with HDAC inhibitor [1].Besides this it can modulate miRNAs(miR-15a, miR-16, miR-21, miR-22, miR-26, miR-101, miR-146, miR-200, miR-203, and let-7) and their multiple taregetgenes [1].In case of breast cancer and leukemia cancer cell line it was found that it upregulates the expression of miR-15a, miR-16 leading to decline the expression level of anti-apoptotic Bcl-2 gene[1].

TABLE 1: Overview of Epigenetic effect of curcumin and their biological incidence in different cancer cell lines

TUMOR TYPE	CELL LINES	TYPES OF EPIGENETIC MODULATION	BIOLOGICAL INCIDENCE	REFERENCE
Breast cancer	MCF 7 cells	DNA methylation	DNA hypomethylation by DNMT1 regulation at mRNA and protein level	[2]
Cervical cancer	HeLa cells	Histone modification	Inhibition of p300/CREB-binding protein(CBP) HAT activity	[3]
	HeLa cells	Histone modification	HDAC inhibition, molecular docking of curcumin with HDAC8	[4]
Prostate cancer	LNcaP cells	DNA methylation	DNA hypomethylation: reversion of CpG methylation of the promoter region of Neurog1/DNMT inhibition	[5]
	PC3 cells	Histone modification	Inhibition of histone hyperacetylation/promotion of proteasome-dependent degradation of p300	[6]

EGCG as an epigenetic modulator:

Epigallocatechin gallate (EGCG)is the ester of epigallocatechin and gallic acid, the most abundant catechin in tea and a major polyphenol in green tea having therapeutic application in the treatment of many diseases like cancer, HIV infectionand studied as a potential demethylating agent.The general formula of EGCG is 5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)chroman-3-yl] 3,4,5-trihydroxybenzoate.

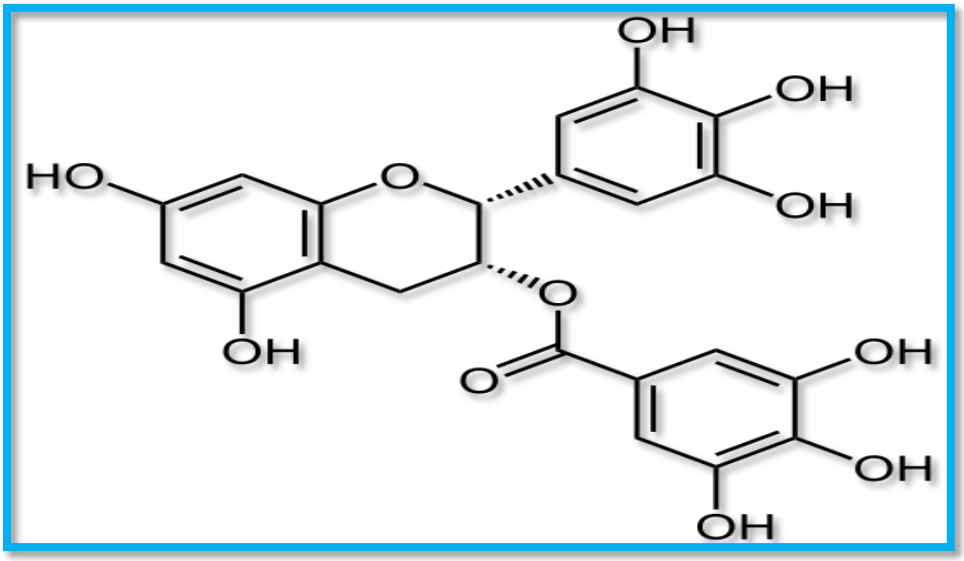


Fig 5: Structure of EGCG

It is the most potent inhibitor of catechol- *O*-methyltransferase activity[7]. Catechol-*O*-methyltransferase and DNMT belong to the same superfamily of *S* adenosylmethionine dependent methyltransferase. At the catalytic site these two enzymes have a common core structure that enhances the possibility of EGCG to inhibit DNMT by binding to a similar catalytic site [8]. Thus it acts as a DNMT inhibitor. It also induces the hypoacetylation of p65by inhibiting the activity of HAT enzymes leading to the down-regulation of NF-κB function by diverse inflammatory signals. Thus EGCG also acts as an inhibitor of HAT [9].Besides these EGCG modulates the expression of miRNA in human hepatocellular carcinoma HepG2 cells [10].

REVIEW OF LITERATURE

RAS GENE MUTATION AND CANCER:

Oncogenic mutation disrupts the signaling cascade and regulatory circuit involved in cell fate, endowing tumor cells to maintain their malignant behavior. It is reported that besides six hallmarks (shown in figure) of cancer cells like self sufficiency or the stimulation in their own growth, insensitive or resistant to inhibitory signals that halts their growth, self ability to limitless replication, tolerate their own programmed death (apoptosis), maintenance of angiogenesis, and tissue invasion (metastasis), metabolic fitness and genomic instabilityalso contribute in tumor malignancy.Cell signaling studies revealed the mechanism which induce oncogenic mutation and maintain the cardinal aberrations.

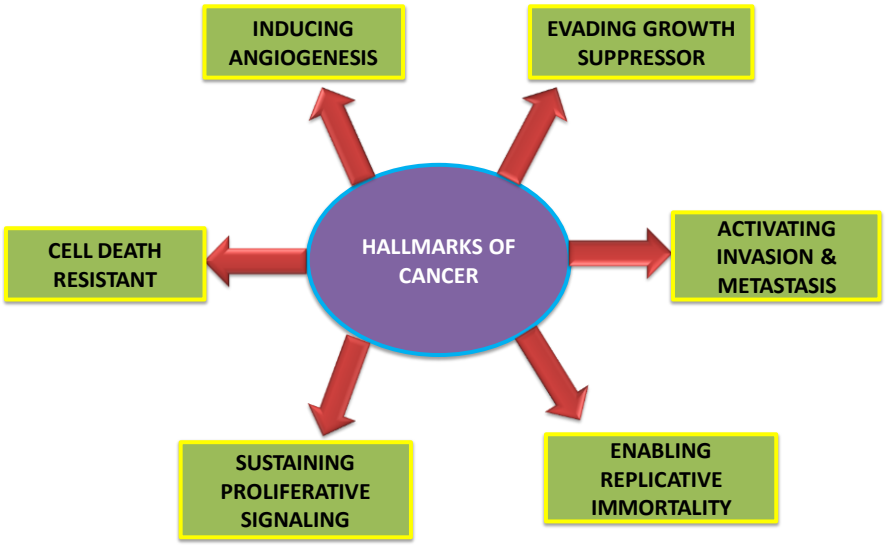


Fig 6: Hallmarks of cancer

RAS proteins are the indispensable components of transduction pathway that controls cellular proliferation, differentiation, or survival. Mutation in any one of the canonical H-Ras, K-

Ras, and N-Ras genes can produce RAS proteins which are permanently activated ultimately causes in cancer.

Upto 30% of all human tumors are caused due to alteration in canonical Ras gene isoform [11]. Oncogenic K-*Ras* mutations being recognized in 25-30% of all screened tumor samples shows that predominantly affect the K-*Ras* locus are predominantly oncogenic mutated [11].This high frequency of K -Ras mutation supports its causative role in human tumorigeneis but the rate of oncogenic mutation in N-Ras and H-Ras were screened in sample much lower i.e 8% and 3% of respectively [11].This data was confirmed and represented by the catalogue of somatic mutations in cancer (COSMIC). In human cancers a great variety of oncogenic *Ras* mutations are found, about 90% of tumors in pancreatic ductal adenocarcinoma were harbored by K-Ras isoform, in contrast bladder, malignant melanoma, thyroid and mammary carcinomaby H-Ras mutation and in hematopoietic tumors K-Ras is frequently mutated [11]. Frequent K- Ras mutations in various carcinomas are quantitatively detected and analyzed that 15%-20% in non-small cell lung carcinoma [12], 40% in colon adenomas [13], 95% in pancreatic ductal adenocarcinoma [14] are caused by K-Ras mutation creating it most common human oncoprotein.

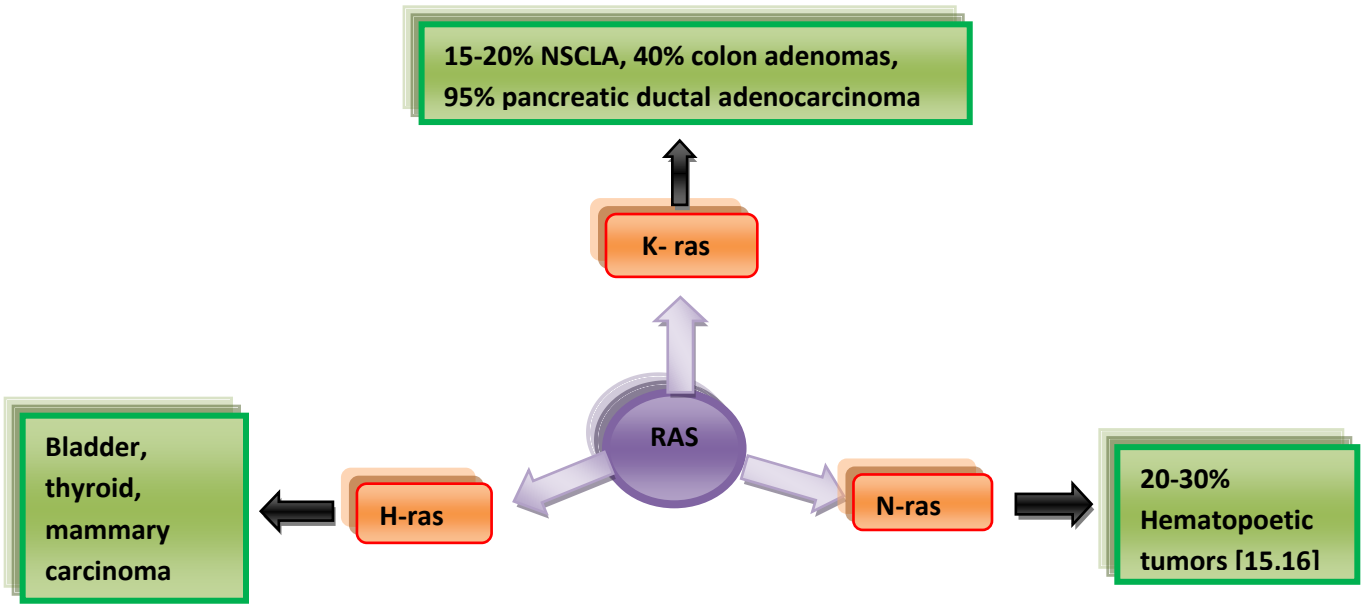


Fig 7: frequency of Ras mutation in human tumors

Codon specificity of Ras isoform mutations:

Codon 12, 13 and 61 of the primary nucleotide sequences of all three isoforms of Ras gene areidentified as three hotspots for oncogenic mutations. Inhibition of GTP hydrolysis due to these codon specific mutation leads to diminish GTPase activity or modulation in guanine exchange [17]. About 99% of K-Ras mutation at codon 12 and very few mutations (about 1%) at codon 61 occurs whereas in N-Ras 35% at codon 12 and 60% at codon 61 was observed and a very high percentage of mutation about 54% at codon 12, 34.4% at codon 61 and 9% at codon 13 in H-Ras was detected [11]. The mutation patterns within the codon are also distinctive. From mutation spectra it is clear that G12D, G12V, Q61K, Q61L and Q61R mutations are predominate over others.G12D is predominately mutated at codon 12 in K-Ras whereas G12V is favored by H-Ras [18]. Aggressiveness of tumors depends on K-Ras mutation. Survival rates of K-Ras G12R andG12A mutations were worst than G12V or G12S mutation in pancreatic ductal adenocarcinoma. In colon cancer K-Ras gene is associated most often in codon 12 (28%) and 13 (8%) on exon 1whereas less frequently at codon 61[19]. Here substitution from Gly to Val in codon 12 has been occurred more frequently in primary metastasis carcinoma suggesting as more aggressive phenotype in colorectal carcinoma [20] but in codon 13 mutation from Gly to Asp has been observed and it shows the reduced survival rate in this cancer type [21].

Isoform specific Ras signaling:

Ras isoforms interacts differentially with the positive and negative regulators of Ras cycle including Ras-GAP110 and RasGEF isoform. It is reported that H-Ras and R-ras but not N-Ras or K- Ras are activated by RasGRF1 [22] whereas in other studies it has been reported that Ras GRP2IS involved in activation of N-ras and K-Ras but not H-Ras[23]. GDP/GTP exchange in all three forms of Ras isoforms i.e. N-Ras, H-Ras,and K-Ras isoform were induced by Sos GEF but have different degree of potency in hierarchy H-RAS> N-Ras> K-Ras [24]. Raf-MEK-ERK kinase cascade and the phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB)/Akt pathway are the most important proliferation inducing signaling cascade that promotes cell survival. Different Ras isoforms act differentially in order to activate these pathways. *In vitro* studies shows that K-Ras has high efficiency to recruit Raf-1 to plasma membrane and thus activates its kinase activity showing that it is the best activator of Raf and Rac whereas H-Ras is

more strongly coupled to PI3K [25]. H-Ras and K-Ras have highest ability to induce and activate NFkB than N-Ras. It has been also reported that cyclopentenone15-deoxy-Δ12,14-prostaglandin can activate H-Ras but not K-Ras and N-Ras by forming covalent adduct which does not occur in K-Ras and N-Ras. So the plethora of cellular responses which is activated by different Ras isoforms can depend on a particular set of effectors which are preferentially activated and the intensity and amplitude of that activation which may itself undergo negative of positive differential modulation. For example it is reported that calmodulin has been involved in the downregulation of Ras-ERK signaling pathway and only K-Ras has the capacity to bind with calmodulin but not H-Ras or N-Ras showing existence of differential mechanism of negative regulation among different Ras isoforms [26]. In NSCLC (non-small cell lung cancer) only 21% mutation in K-Ras gene in codon 12 and 13 were identified [27] but in case of breast cancer Hollestelle at al. found only 12.5% [28] mutations whereas Sanger COSMIC database version 28 (http://www.sanger.ac.uk/genetics/CGP/cosmic/) records only a 5% [29].From this it was analyzed that K-Ras are less frequently mutated, suggesting that K-Ras gene mutation is least important in breast cancer carcinogenesis as compared to other forms of cancer.

K-Ras in human cancer:

The K-Ras gene (Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) is an oncogene encoding K-Ras which is a small GTPase transductor protein which is involved in the cell cycle regulation. It contains 188 amino acids having molecular weight 21.6kD. KRAS1 and KRAS2 are two copies of K-Ras gene in human genome located at chromosomes 6 and 12 respectively [30]. An allelic loss of chromosome 12p12-13 region and KRAS gene mutation generally occur in human cancer[31]. K-Ras 1 is a pseudogene which is derived from KRAS2 by alternate mRNA splicing hence it is officially known as KRAS1P [32]. KRAS gene has 6 exons out of which 2, 3 and 4 are invariant coding exon. During alternate splicing exon 5 can be skipped, generating two isoforms KRASA and KRASB whereas 6^{th} exon C terminal region in KRASB but untranslated in KRASA. This protein is in inactive state until it interacts with GTP. Any mutations in the K-Ras gene weaken the capability of the K-Ras protein to switch between active and inactive states results in cell transformation and raise the resistance to chemotherapy and biological therapies which targets the receptors of epidermal growth factor.Usually wild type K-Ras promotes cell cycle progression acting as tumor suppressor but mutation in this gene

increases the abnormal level resulting in acquiring its oncogenic properties and seems to be involved in the development various tpes of cancer in humans. Oncogenic mutation prevents GTP hydrolysis causing the activation of RAS molecules permanently. Mutated K-Ras gene expression in fibroblasts results in augment of expression of metalloproteinase 2 (MMP2) in the matrix which ultimately enhance the cancer cell invasion [33]. Overexpression of mutated form of K-Ras cause the inhibition of glycosylation of the intigrinβ1 chain causes the alternation in polarization and hence enhances the adhesiveness of colon cancer cells [34].

K-Ras gene mutation have important role in carcinogenesis depending upon the cells and tissue involved. In cancer cells these mutation frequently occurs on position 12 and 13 in exon 1 and less frequently in codon 61, 63,117,119, and 146 [35, 36]. Mutation in codon 12 of K-Ras gene causes the encoded protein "ice up" in its active state for much more longer period of time than in non- mutated form resulting in substitution in amino acids 116,117, 119and 146 causing the reduction of nucleotide affinity of K-Ras protein, thereby affecting the GDP/GTP exchange rate [34]. Overexpression of mutant K-Rasallele can be induced by loss of p16INK4 (CDKN2A), p19INK4 (CDKN2D), or p53 [37] and deletion of wild type allele results in transforming activity of KRAS oncogene.

PI3/ AKT signaling cascade in tumor progression:

Normal mammary epithelial cells transformed into cancer cells in a multistep development with alteration in signaling cascade conferring an imperative survival and growth advantages to malignant cells. Phosphatidylinositol 3-kinase (PI3K) pathway is a key mediator of cell growth andits metabolism, affected bygenetic alterations at different stages becoming a crucial cascade for cancer progression which outcome in a therapeutictarget which can act against breast cancer. This pathway is the most frequently mutated and genetically altered in excess of 70% of breast cancer with the amplification or alteration of genes which encodes PI3 catalytic subunit p110α (*PIK3CA*) and p110β (*PIK3CB*), the PI3K regulatory subunit p85α (*PIK3R1*), receptor tyrosine kinase such as HGR2, ERBB2 and fibroblast growth factor receptor 1, the PI3K activator K-Ras, the PI3K effectors AKT1, AKT2, and phosphoinositide- dependent kinase 1 (PDK1), and loss of the lipid phosphatase PTEN (phosphatase and tensin homo log) and

INPP4B (inositol polyphosphate-4-phosphatase, type II [38]. These genetic alterations in various components of PI3K signaling cascade are summarized in table 2.

Table 2: Genetic alteration in PI3K signaling Pathway

GENE	MODIFICATIONS	COMMENTS	CANCER TYPE	REFERENCES
PTEN	Loss of function by somatic mutation	Truncation, loss of phosphatase activity	Bladder, brain, breast, cervical, colorectal, endometrial, gastric, head and neck, kidney	COSMIC, [39]
	Epigenetic silencing	Transcriptional repression by promoter hypermethylation	Breast, colon, melanoma	[40], [41], [42]
PIK3CA	Gain of functionby somatic mutation	Exon 9 helical domain and exon 20 catalytic domain.	Breast, colorectal, glioblastoma, endometrial, cervical, lymphoma, ovarian, pancreatic, prostate, thyroid	COSMIC [43], [44]
	Amplification	Increased protein level and its activity	Breast, cervical, gastric, lung, ovarian, prostate	[45], [46], [47]
AKT1	Gain of function by somatic mutation	Pleckstrin homology domain, membrane localization, and constitutive activation	Breast, colon, endometrial, melanoma, ovarian	[48], [49]

AKT2	Gain of function by somatic mutation, amplification	Kinase domain mutation	colorectal	[50], [51]
AKT3	Gain of function by somatic mutation	Pleckstrin homology domain, membrane localization, and constitutive activation	melanoma	[49]
PDK1	Gain of function by somatic mutation	Kinase domain mutation	colorectal	[50]

PTEN works antagonistically to PI3K. I possesses protein tyrosine phosphatase activity and lipid phosphatase capable to cleave 3'phosphate group from PI(3,4,5)P3 which is necessary to function as tumor suppressor. Inactivation of PTEN tumor suppressor gene is the most common genetic modification in the PI3 signaling cascade leading to the loss of lipid phosphatase activity which cause the accumulation of PIP3 [53,54]. Homozygous and hemizygous deletion of PTEN are seen in many human cancer cases. Transcriptional repression and epigenetic silencing of PTEN through hypermethylation at promoter region results in PTEN inactivation [40,41]. At present somatic mutation in PIK3CA has been studied in various different forms of cancers like breast, colon, endometrial cancer and glioblastomas. Exon 9 and 20 are the two hot spots for these mutations [43,44]. The catalytic domain of p110α is encoded by exon 20 and any type of mutation in this domain may constitutively activate its enzymatic activity whereas the helical domain of p110α is encoded by exon 9 and mutations at this region can de-repress an inhibitory interaction between the N-terminal SH2domain of p85 and the p110 α catalytic subunit [55,56]. Expression of these *PIK3CA* mutants leads to raised oncogenic potential in vitro and in vivo,resulting in constitutive signaling along the PI3K pathway in the absence of growth factors and therefore seems to obviate the usual obligate communications with tyrosine phosphorylated RTKsand/or adapters [57]. Mutation in p85 regulatory subunit PIK3R1 are also identified in various human cancer forms including colorectal cancer, glioblastomas, ovarian cancers resulting in truncation or in frame shift deletion on inter- SH2 domain of

p85α[57]. AKT family including Akt1, Akt2 and Akt3 are also undergo mutation and were recognized in human cancer. A single amino acid replacement, E17K on PH domain of Akt1 was identified in breast, endometrial, ovarian, colorectal cancers as well as melanomas whereas E17K mutation in Akt3 was identified in melanomas [58].

PI3K activates downstream RTK signaling in normal epithelial cells but often mutated, overexpressed or amplified resulting in aberrant activation of PI3K in cancerous cells. The activation of PI3K by epidermal growth factor receptor (EGFR) in lung cancer harbors somatic activating mutations in EGFR and by human epidermal growth factor receptor 2 (HER2) in breast cancer with HER2 amplification [59].

PI3 pathway AND breast Cancer Subtype:

Luminal A, luminal B, HER2-enriched, and basal like tumors are sub types of breast cancer are grouped on the basis of their gene expression and the frequency of aberration of PI3K varies among these different sub types. Upto 40% of PIK3CA mutations have been founds in hormone receptor positive breast cancer showing the most frequent aberration in PI3K pathway. Here the mutation is associated with mTORC1 signaling. Mutations due to Akt activation are linked to preliminary tumorigenesis with posterior inhibition of invasion and metastasis. Enhanced PI3K activities were found in basal- like tumors i.e., triple negative for ER, PR and HER2 through PTEN loss [60]. 30% PTEN loss were recorded in basal- like breast cancer and cause MEK inhibition [61]. In 20-25% of human breast cancer HER2 are amplified mainly through the PTEN loss [60]. In ES cells and in embryonic fibroblast PTEN is inactive resulting in increased level of PIP3 [62]. Deficiency of PTEN led to enhance the phosphorylation and activation of Akt/PKB pathway ultimately increase the phosphorylation of BAD and promoted PTEN (-/-) cell survival [63]

Clinical uses of PI3K cascade inhibitors:

The upstream and downstream effectors of PI3k pathway comprise a potential target for drug development in breast cancer. Agents inhibiting this pathway at any level unaccompanied or in combination with chemotherapy, radiations are clinically used. Wortmannin and LY294002 are two best inhibitors.Wortmannin derived from *Penicilliumwortmannin*is a natural inhibitor which binds irreversibly to PI3K enzymes resulting in covalent modification in lysine, compulsory for

catalytic activity whereas LY294002 is a synthetic drug capable of reversibly targeting PIP3 family helps in decreasing proliferation and increasing apoptosis and in conjugation with Arg-Gly-Asp peptides are used as multimodal Pan-Pi3k inhibitor [60]. In a study ita was found thatA constitutively active Ras mutant cells were radiosensitized by PI3K inhibitor LY294002 but does not any affect on the survival of cells with wild-type Ras suggesting that AKT may be a potential target for raising the response to radiotherapy in breast cancer patients [64]. GSK690693 (GlaxoSmithKline) is an ATP-competitive AKT kinase inhibitor thattargets all three isoforms of AKT. Reconstitution PTEN reduces AKT phosphorylation hence induces the transactivation of p53 resulting in increase the p53 target gene expression in glioma cells [65]. Thus PTEN and LY294002 affected p53 activity in endothelial cells of human brain, suggests that they can suppress the cancer progression directly on tumor and endothelial cells and block tumor progression *in vivo* [65].

OBJECTIVES

1. To determine the effect of natural polyphenols like CURCUMIN and Epigallocatechin gallate (EGCG) on breast cancer cell line MD-MB-231.

2. To analyze the change in gene expression of K-Ras and Akt1 in presence of curcumin and EGCG.

3. To analyze the apoptosis inducing propensity of these agents in breast cancer cell line MDA-MB-231.

4. To observe the cell survivability and proliferating capacity of MDA-MB-231 cell line in presence of curcumin and EGCG.

METHODS AND MATERIALS

1) CELL LINES AND CELL CULTURE:

We obtained the MDA MB-231 cell line from the *National Centre for Cell Science (NCCS), Pune, India.* The cells are known to be of epithelial breast adenocarcinoma origin, adherent and are triple negative. Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 2 mM L-glutamine, and 100 units/ml penicillin-streptomycin sulfate (Invitrogen) was used to maintain cells growth. First cells were washed with 1 ml of 1X PBS then PBS was decanted then 500 µl of trypsine was added and left for 15 min. cells were observed under microscope. After that 1.5 ml of media was added. Cells were counted by using hemocytometer under microscope.Desired amount of cells were seeded. A treatment was carried out with curcumin and EGCG, all purchased from Sigma and added to the regular growth media under sterile conditions. These cells were cultured in a 37°C, 5% CO_2 incubator.

2) MTT ASSAY:

Cell viability analysis and cytotoxicity studies were performed using the MTT assay based on the observation that the mitochondria in living cells can catalyze MTT molecules to a colorimetrically detectable dye. MTT assay works on the principle that yellow colored MTT is reduced to purple colored *formazan* in the mitochondria of living cells by active mitochondrial reductase enzyme.To determine the proliferative activity, MDA MB-231 cells were seeded in three 96-well plates at a density (3000 cells/well) based on the doubling time, with 200 µl growth media (10% FBS) and incubate for 24, 48 and 72 hrs in incubator with 5% CO_2 concentration at 37ºC. Cell seeding must be uniform in order to obtain a dose response effect of the drug. After 24 hours existing media was removed, washed with PBS and replaced with media with various concentration of drugs (5-50µM for curcumin and 50-300µMfor EGCG) and was incubated for 24, 48 and 72 hours at 37º C. MTT working solution was prepared having 0.8mg/ml. 100 µl as final concentration to detect the cell viability by diluting the stock solution (stock 5mg/ml PBS, PH 7.2) in growth medium without FBS. MTT working solution was added to each well and incubated for 4 hours in CO_2 incubator. The media was removed carefully after incubation without disturbing *formazan* precipitate and 100 µl of 100% DMSOwas added to it. An

incubation of 25 minutes was carried out in dark and the colorimetric estimation of *formazan* product was performed at 570nm in an ELISA reader. The data was plotted against drug to calculate the optimal growth inhibitory concentration (IC_{50}) of the drugs.

3) ISOLATION OF TOTAL CELLULAR RNA

The total cellular RNA was extracted from MDAMB-231cells using TRI reagent (Sigma), following the manufacturer's instructions. The drug treated cells (10^5 cells) were washed with 1 ml ice cold PBS and then trypsinized. 1ml of Tri reagent was added in a culture dish. After addition of the reagent, a homogenous lysate was formed by pipetting cell lysate several times. These samples were allowed to stand for 5 minutes at room temperature so that complete dissociation of nucleoprotein complexes takes place. 0.2ml of chloroform was added for per ml of TRI Reagent used,vigorously shaken for 15 seconds and incubated for 2–15 minutes at room temperature. This mixture was then centrifuged at 12,000 g for 15 minutes at 4ºC to separate the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA).The upper aqueous phase was transferred to a fresh tube without disturbing interphase and 0.5 ml of isopropanol was added per ml of TRI Reagent and mixed. The sample was allowed to stand for 5– 10 minutes at room temperature and centrifuged at 12,000 g for 10 minutes at 4ºC. The RNA precipitate formed a pellet on the side and bottom of the tube. The supernatant was removed and 1 ml of 75% ethanol per 1 ml of TRI Reagent was added to wash RNA pellets. The sample were mixed by vortexing and then centrifuged at 7,500 g for 5 minutes at 4ºC. Then the RNA pellets were briefly dried for 5–10 minutes by air drying. 40µl of DEPC treated water was added and mixed by repeated tapping. The RNA was stored at - 20° C for further use or immediately processed for cDNA synthesis.

4) QUANTIFICATION OF THE TOTAL CELLULAR RNA

Final preparation of RNA was analyzed using a nano-drop UV spectrophotometric analyzer. For analysis 2µl of RNA mixed with DEPC water was taken and put on nano drop cuvette. 260/280 and 260/230 ratio was noted. It was likely that a standard preparation of RNA should have a 260/280 ratio of 1.8-2.0 and a 260/230 ratio of less than 260/280 ratio which indicates the

preparation to be free from proteins and oligo-peptides contamination The extracted RNA was run on a denaturing agarose gel and the quantity of RNA estimated from the band intensity.

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5) cDNA SYNTHESIS AND EVALUATION:

First strand cDNA was synthesized using Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit. In PCR tube 1µl of oligo (dT)18 primer,1 µg of total RNA(drug treated) was taken and the volume was made up to 12 µl with the addition of nuclease free water. The mixture was gently mixed with pipetting and was briefly centrifuged. Then the mixture was incubated at 65°C for 5 minutes. After 5 minutes, the tubes containing mixture was snap cooled on ice. Then to this 12 µl mixture, 4 µl of 5X Reaction Buffer, 1 µl of RiboLock RNase Inhibitor (20 µg/ µl) ,2 µl of 10 mM dNTP Mixture and1 µl of RevertAid M-MuLV Reverse Transcriptase (200 µg/ µl) was added. The total volume of the mixture now became 20 µl. All the steps were performed while kept on ice. The total mixture was mixed thoroughly with gentle pipetting and spins down for few seconds to ensure proper mixing. Then the mixture was set for reverse transcriptase-PCR with incubation at 42°C for 60 minutes followed by end incubation at 70°C for 5 minutes. The cDNA synthesized from the RNA was stored in ice at -20°C for further use.

6) qRT-PCR ANALYSIS

Real time Polymerase Chain Reaction or qRT- PCR is a method that allows exponential amplification of DNA sequences and simultaneously quantitates differences in mRNA expression. This system includes a DNA binding cyanine dye such as SYBR green that specifically binds to the major groove of double strand DNA but not to the single stranded DNA. SYBR green binds to the amplicons accumulated during PCR process which is proportional to the fluorescence emission of the dye. One reference dye (ROXdye) was used to serve as an internal reference for normalization of the SYBR green fluorescent signals.ROX dye allows for correction of well-to-well variation due to pipetting inaccuracies and fluorescence fluctuations. Four genes were taken for the experiment, three of which were test genes and one was housekeeping gene as a reference. Housekeeping gene are generally taken as reference genes to check for the expression of test genes i.e. how much fold the test gene's expression has increased

or decreased with respect to normal gene expression. For this experiment the test genes were K-Ras, Akt1 and reference gene used was β-actin.

The total reaction volume prepared was 5 µl.

Calculation:

3genes were taken, therefore,

3 genes x 3 replicates×2 treatment = 18 reactions~20 reactions (to avoid inaccuracy due to pipette error) (12 reactions for each treatment)

20 reactions x 5 µl = 100µl total volume was to be prepared.

SYBR ® Green master mix dilution:

The stock SYBR ® Green master mix solution was of 2X concentration(containing optimized mixture of SYBR green dye+$MgCl_2$ +dNTPs+ Taq DNA Polymerase+ qPCR Reaction buffer) and from this working solution of 1 X concentration was taken.

2X x (? µl) = 1X x 100µl

=> (? µl) = 1X x 100µl / 2X

= 50µl

SYBR ® Green master mix= 50µl

Reference dye:

For 100 µl of mixture reference dye taken, is 1 µl.

Hence for 130 µl reaction mixture reference dye taken was,100 µl/100=1 µl

cDNA was diluted at 1:20 ratio with addition of nuclease free water.

For each reaction we required 1µl of diluted cDNA (EGCG treated and curcumin treated).

Therefore, for 20 reaction= 1x20=20µl (for each treatment 20 µl template was needed.)

Primer:

The stock solution of primer contained 10µM, from which we required 500nM for each reaction

10 µM x (? µl) = 500x10^{-3}µM x100

=> (? µl) = 500x10^{-3}µM x100 / 10 µM

= 5µl(each for forward and reverse primer)

Reference dye=1 µl

Forward Primer=5 µl

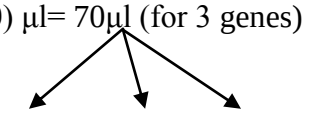
Reverse primer= 5 µ

Template=20 µl

Autoclaved distilled water = 19µl

Total= 50µl

Then a semi master mix was prepared with the addition of SYBR® Green master mix + Reference dye +water= (50 +1+19) µl= 70µl (for 3 genes)

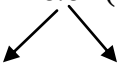


23.33µl 23.33µl23.33µl

Forward Primer= +1.67

Reverse Primer= +1.67

Total= 26.67 (for 2 treatment)

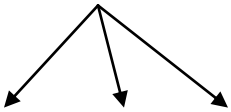


13.34µl13.34µl(for 5 µl of reaction 1 µl template is needed,

For 13.34µl of reaction template needed= 13.34/5=2.67)

Template= + 2.67+ 2.67

Total= 16.01 µl (for each triplicate)



5.33 µl 5.33 µl 5.33 µl

For Real time PCR analysis mainly 2 types of primers are used: Forward primer and reverse primer. The primers were specifically designed with the help of NCBI primer blast tool.The primer sequence, their melting temperature and product length is shown below in table no.3:

TABLE 3: Table showing the sequence of the forward and reverse primers

NAME OF THE PRIMER	**TYPE**	**SEQUENCE**	**PRODUCT LENGTH**
K-RAS	Forward	5'ACTGGGGAGGGCTTTCTTTG3'	20
	Reverse	5'GGCATCATCAACACCCTGTCT3'	21
AKT1	Forward	5’ACCTCTGAGACTGACACCATG3’	21
	Reverse	5’CACTGGCTGAGTAGGAGAAC3’	20
β- actin	Forward	5’CTGGAACGGTGAAGGTGACA3’	20
	Reverse	5’AAGGGACTTCCTGTAACAACGCA3’	23

Real-time PCR was carried out in Eppendorf Masterplex Real Time PCR. The experiment was set up with the following PCR program. The threshold frequency taken was 33%. The cycle temperatures taken were as follows:

Table 4: Cycle temperature and time for qRT-PCR

Stage	Temperature (°C)	Time	Cycle
Stage 1	95	20sec	1
Stage 2	95 55 68	15sec 15sec 20sec	40
Stage 3	95 60 95	15sec 15sec 15sec	1

The melting curve was analysed by the inbuilt software and the change in relative gene expression with respect to β actin was detcted as fold change at logarithmic scale.

7) ANALYSIS OF CHROMATIN CONDENSATION BY *HOECHST 33342* STAIN

About 1 X 10^4 cells were seeded in a Petri-plate and after 24 hours of seeding they were treated with curcumin and EGCG. After 24 hours of drug treatment treated cells were stained with 1 mg/ml of Hoechst 33342 stains and allowed to incubate for 10 min at 37°C and the images were taken under UV filter using Epi-fluorescent Microscope (Nikon TE 2000E) at 400X magnification with an excitation wavelength of 355-366 nm and an emission wavelength of 465-480 nm. To analyze the percentages of apoptotic nuclei, condensed nucleus was counted against total number of nucleus in the field.

8) COMET ASSAY TO MEASURE THE DNA DAMAGE

Comet assay is a gel electrophoresis with fluorescence microscopy based method used to visualize migration of DNA strands and to measure DNA damage from individual eukaryotic cells.Circular comet head contains undamaged DNA having high molecular weight and the comet tail represents damaged DNA. Longer and brighter tail shows higher level of DNA damage.

8.1) Agarose preparation

Two water baths were equilibrated at 40 °C and 100 °C respectively. Than 1% low gelling-temperature agarose was prepared by mixing powdered agarose with distilled water in a glassbeaker or bottle. The bottle was placed in the 100 °C water bath for several minutes and was transferred into a 40 °C water bath.

8.2) Slide Precoating

Agarose-precoated slides were prepared by dipping the slides into molten 1% agarose and wiping one side clean. It is best to work in a low-humidity environment to ensure agarose adhesion. Agarose was allowed to air-dry to a thin film. Slides can be prepared ahead of time and stored with desiccant.

8.3) Sample Preparation

A single-cell suspension was prepared using enzyme disaggregation or mechanical dissociation. The cells were kept in ice-cold medium or phosphate-buffered saline to minimize cell aggregation and inhibit DNA repair. Using a hemocytometer or particle counter, cell density was adjusted to about 2×10^4cells/ml in phosphate-buffered saline lacking divalent cations. Slides were labeled on frosted end using a pencil. 0.4 ml of cells into a 5 ml plastic disposable tube was pipette out. 1.2 ml 1% low-gelling-temperature agarose at was added at 40 °C. 1.2 ml of cell suspension onto the agarose-covered surface of a pre-coated slide was mixed by vigorous pipetting. Agarose was allowed to be gel for about 2 min.

8.4) Lysis and Electrophoresis

After agarose has gelled, slides were submerged in a covered dish containing A1 lysis solution [1.2M NaCl, 100mM Na_2EDTA, 0.1% sodium lauryl sarcosinate, 0.26M NaOH

(pH.13)]. Samples were lysed overnight (18−20 h) at 4 °C in the dark. After overnight lysis, slides were removed carefully and submerge in A2 rinse solution [0.03M NaOH, 2mM na_2EDTA (pH 12.3)] for 20 min at room temperature (18−25 °C). The process was repeated two times to ensure removal of salt and detergent. Care was taken for not allowing DNA to renature even briefly (i.e., by lowering pH below 12.3) until after electrophoresis, as this will result in DNA tangling and reduced migration. After these three rinses, slides were submerged in fresh A2 solution in an electrophoresis chamber. The chamber was filled with a consistent volume of buffer that is about 1–2 mm above the top of the agarose. It was ensured that the chamber is level using a bubble leveling device. Electrophoresis was conducted in solution A2 for 25 min at a voltage of 0.6 V/cm. The current was about 40 mA using 20 V.

8.5) Slide Staining

Slides were removed from electrophoresis chamber and were rinsed and neutralizedin 400 ml of distilled water. Slides were placed in staining solution containing 2.5 µg/ml of propidium iodide in distilled water for 20 min. Finally the slides were rinsed with 400 ml distilled water to remove excess stain.

8.6) Slide Analysis

Analyses of cells were done by examining at least 50 comet images from each slide. Analyzing doublets or comets at slide edges should be avoided. Image analysis software was used to analyze individual comet images.

9) DNA FRAGMENTATION ASSAY

Culture media were collected and 1ml of trypsin was added to monolayer on 100mm dishes, cells were then scrapped and harvested (culture media and cell monolayer) by centrifugation at 2,500 rpm for 5 min. Cell pellets were then washed with 1X PBS. 100 µl of lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5) was added and kept for 10 sec in order to lyse the cells. For the Preparation of 1µl of lysis buffer 10% NP-40 100 ml, 200 mM EDTA 100 µl , 0.2 M Tris-HCl (pH 7.5) 250 µl , D.W. 550 µl were added together. After adding lysis buffer the solution was centrifuged at 3,000 rpm for 5 min to obtain the supernatant 10 µl of 10% SDS

solution was added to pooled supernatant (final: 1% SDS),then it was treated with 10 µl of 50 mg/ml RNase A (final 5 mg/ml) and incubated for 2 h at 56 °C. after that 10 µl of 25 mg/ml Proteinase K (final 2.5 mg/ml) was added and again incubated for 2 h at 37 °C. To this 1/2 vol. (65 µl) of 10 M ammonium acetate was added. 2.5 vol. (500 µl) of ice-cold ethanol was then added and mixed thoroughly and allowed to stand for 1 h in – 80 °C freezer so that ethanol was precipitated. The solution was centrifuge for 20 min at 12,000 rpm, and then the white pellet was washed with 200 µl 80% ice cold ethanol and allowedto air-dry for 10 min at room temperature. The pellet was dissolved with 50 µl of TE buffer. DNA concentration was determined by taking absorbance at 260 and about 4 µg of the same concentration of DNA was run in 2% agarose gel electrophoresis.

10) COLONOGENIC CELL SURVIVAL ASSAY

The colonogenic cell survival assay determines the capacity of cells to produce its clone. Usually from a single cell a colony of 50 or more cells are formed. Single cell suspensions were prepared by trypsinization. Then these cells were washed with phosphate buffered saline and incubated with a 0.05% trypsin 5-10 minutes. After that 1.5 ml of Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum is added to neutralize the trypsin. The cells were detached by pipetting up and down. Cells were counted using a hemocytometer. These cells were treated with different drugs like curcumin and EGCG having 25 µl and 200 µl concentration respectively and then seeded in triplicate into petri-dishes with densities varying from 600-1000 cells/ dish so that we get 50–200 colonies/dish. The cells were then culturedin a 37°C, 5% CO_2 incubator for 7-10 days. Then slides were dipped in 1 ml of 1X cold PBS and kept for 10 min followed by dipping in 1 ml absolute cold methanol for 10 min. Finally the clones were stained with 0.05% crystal violet and kept for 10-15 min and then destained by washing with water for 4 times. These slides were observed under Epi- fluorescent microscope (Nikon TE 2000E) at 400X magnification with an excitation wavelength of 355-366 nm and an emission wavelength of 465-480 nm.

11) SOFT AGAR ASSAY

It is an anchorage independent growth assay used to detect malignant transformation of cells.

10.1) Preparation of base agar

0.7% agar was melted in a microwave. Then 2X DMEM with 20% FBS and antibiotics was taken in a falcon tube. Equal volumes of these two solutions were mixed to get 0.7%Agar+1X DMEM+ 10% FBS. From this mixture 1.5 ml was taken and added to each 35mm petri dish and set aside for 5-10 min to allow agar to solidify.

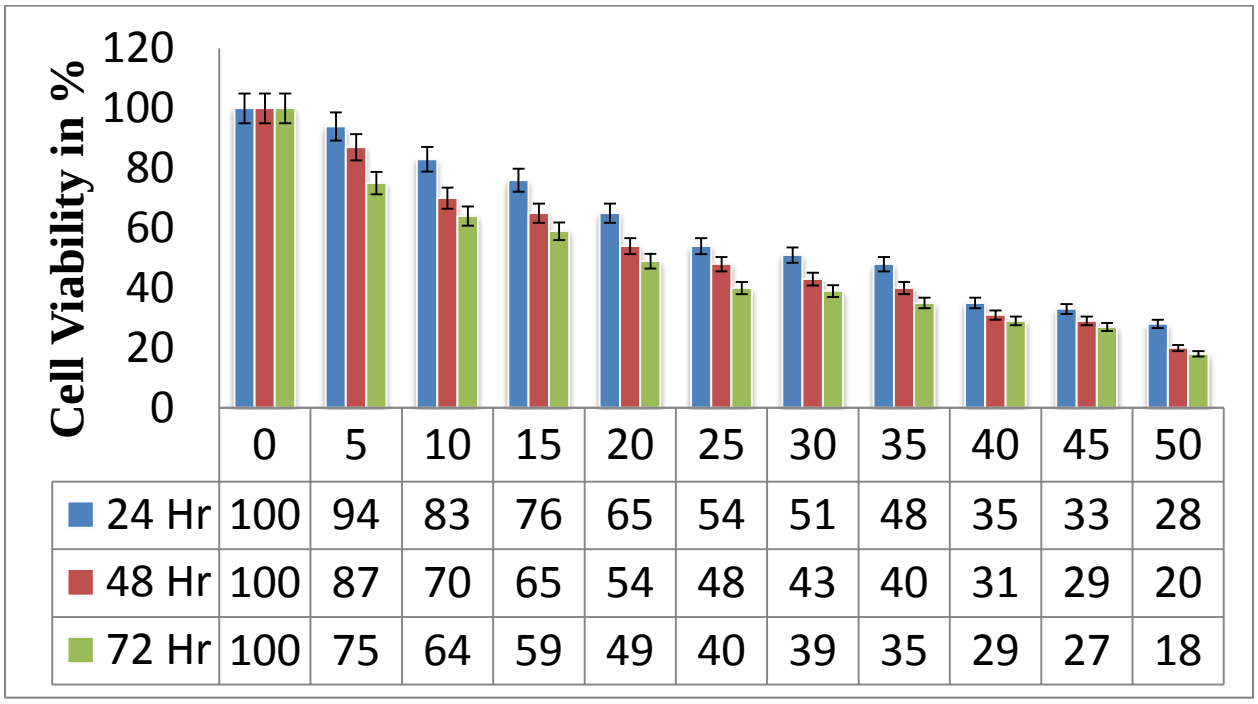
10.2) Preparation of top agarose

0.8% Agarose was melted in a microwave. 2X DMEM with 20% FBS was taken in a falcon.The adherent cells were trypsinized to release them and number of cells per ml was counted. 5,000 cells /plate were required for each 35 mm Petri dishes. These cells were then treated with different drugs like curcumin and EGCG. 0.1ml of cell suspension was added to 10ml tubes. 35mm base agar dishes were labeled appropriately. 3 ml of 2X DMEM +10%or 20% FBS and 3 ml of 0.7% agarose was added to a tube of cells for plating. They were mixed gently by swirling and 1.5 ml was added to each of the three replicate plates. Then these plates were incubated at 37 ºC in a humidified incubator for 10 to 20 days. The cells were fed 1-2 times per week with cell culture media. After 10-20 days plates were stained with 0.5 ml of 0.005% of crystal violet for more than 1 hour. After staining colonies were counted using a dissecting microscope.

RESULT AND DISCUSSION

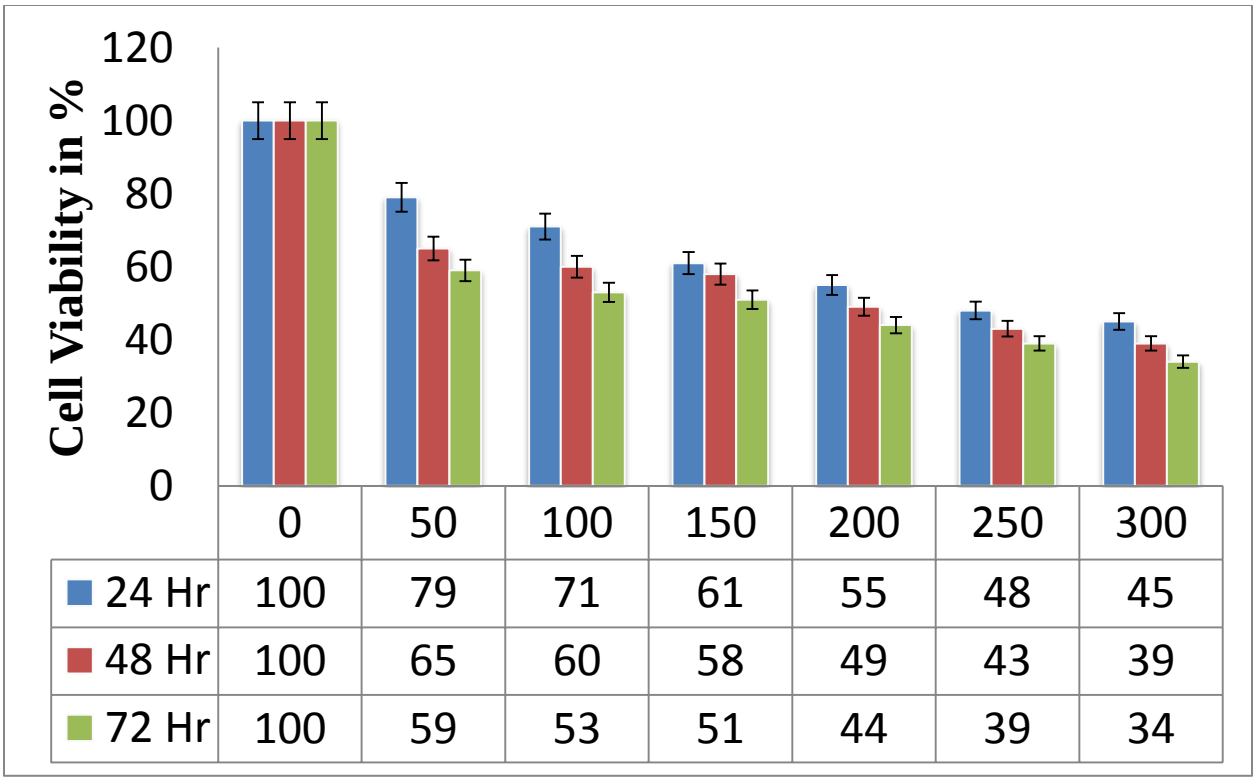
1) CELL VIABILITYASSAYBY MTTMETHOD

Breast cancer cell line MDA MB-231 viability after the treatment of drugs like curcumin and EGCG was determined by doing MTT assay. In order to determine the optimum dosage, different concentrations of the drugs were considered and the treatment was done for different time intervals i.e., 24 hours, 48 hours and 72 hours. The IC_{50} value for the both drug were calculated.The result obtained from MTT assay is shown in figure 8 and 9:



	0	5	10	15	20	25	30	35	40	45	50
24 Hr	100	94	83	76	65	54	51	48	35	33	28
48 Hr	100	87	70	65	54	48	43	40	31	29	20
72 Hr	100	75	64	59	49	40	39	35	29	27	18

Fig 8: Graphical representation of curcumin treated MDA-MB-231cells



	0	50	100	150	200	250	300
24 Hr	100	79	71	61	55	48	45
48 Hr	100	65	60	58	49	43	39
72 Hr	100	59	53	51	44	39	34

Fig 9: Graphical representation of EGCG treated MDA-MB-231cells

From this graphical data it can be inferred that cell viability declined progressively with an increased dose of natural polyphenols, curcumin and EGCG.From this assay 25 µM of curcumin and 200 µM of EGCG can be taken as optimum dose for further analysis.

2) ISOLATION OF TOTAL CELLULAR RNA BY TRIZOL METHOD

The total cellular RNA was isolated following the manufacturer's instructions by Tri-reagent (Sigma). The isolation was almost pure and in good yield as the reading in Nanodrop spectrophotometer showed the 260/280 absorption ratio was 2.01 and the 260/230 absorption ratio above 1.8 and the 260/230 absorption ratio was less than 260/280 ratio for all the samples quantified. The concentration and ratio for both drug treated cells were summarized in the table given below:

TABLE 5: RNA concentration and ratio for the control and drug treated cells

SAMPLE	CONCENTRATION (µg/ml)	260/280 RATIO	260/230 RATIO
control	909.3	1.9	1.91
Curcumin treated cells	617.0	2.01	2.05
EGCG treated cells	272.9	1.96	1.42

3) cDNA SYNTHESIS AND qRT-PCR

In general qRT-PCR data confirmed that K-Ras and Akt1 genes are upregulated in breast cancer cell line MDA MB-231. But when these cells were treated with curcumin and EGCG their expression was downregulated.

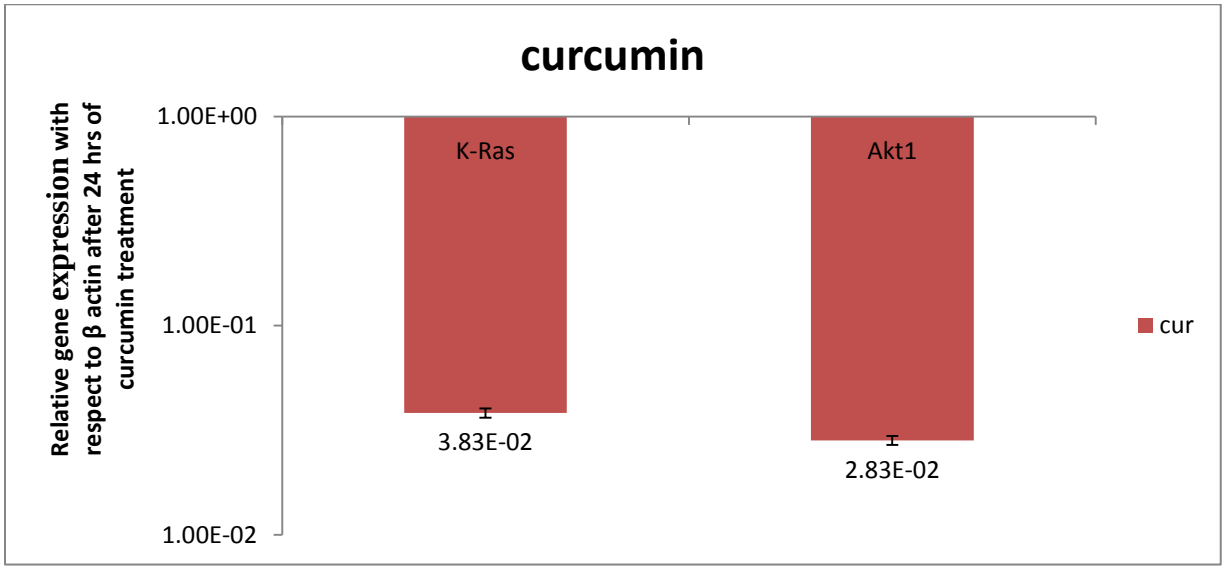


Fig 10:RT-PCR analysis of K-Ras and Akt-1 in presence of curcumin in MDA-MB 231 Cell line

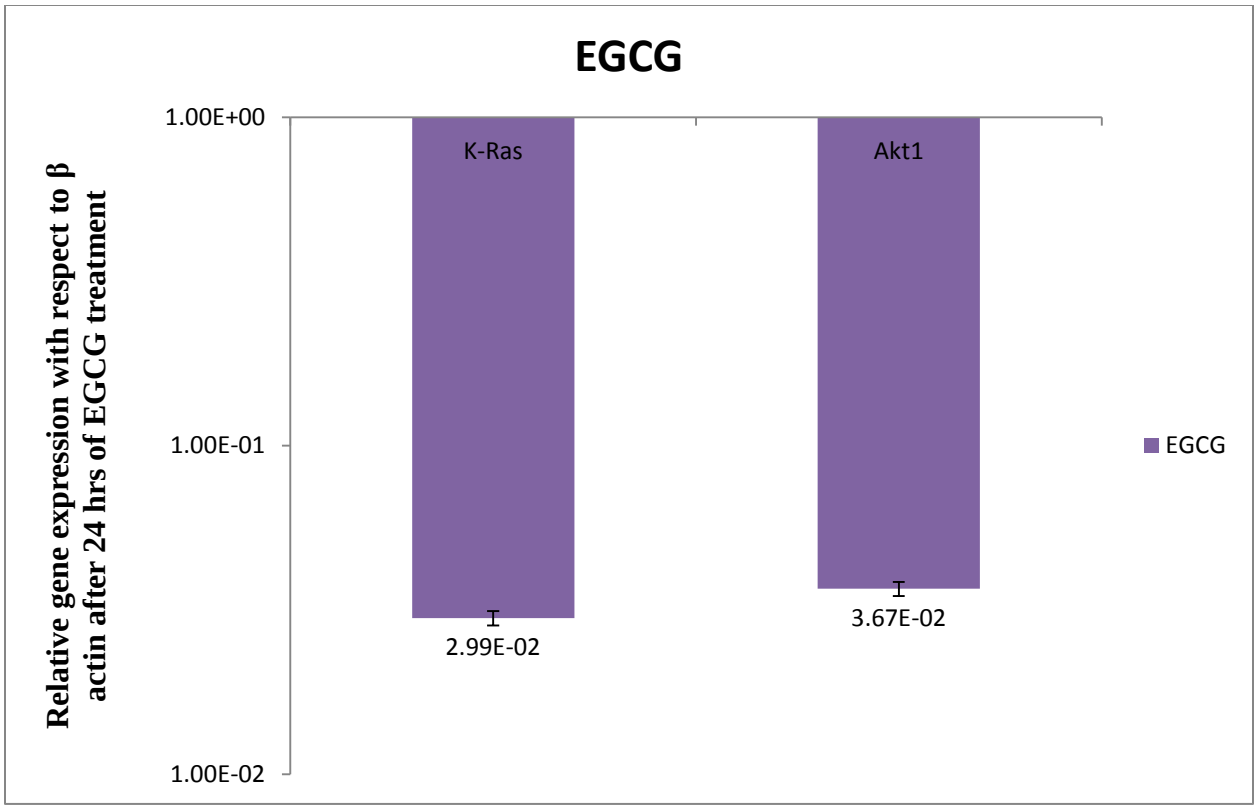
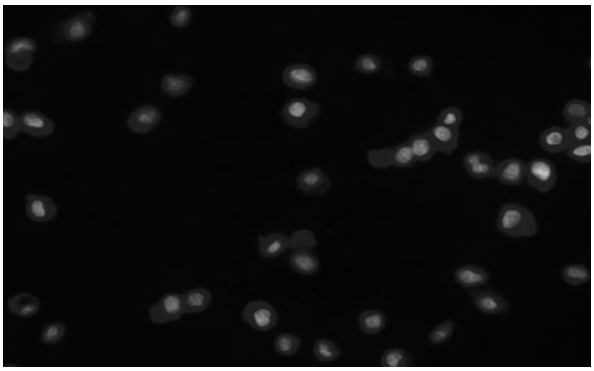


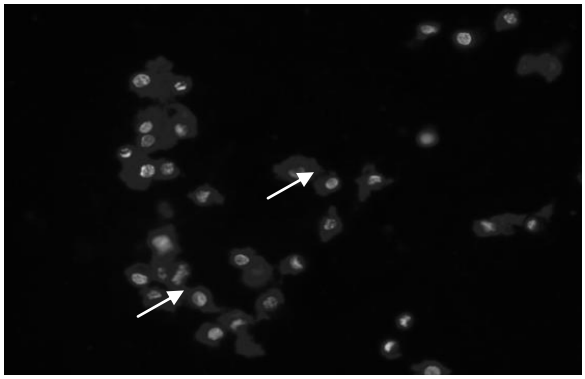
Fig 11: RT-PCR analysis of K-Ras and Akt-1 in presence of EGCG in MDA-MB 231 Cell line

4) ANALYSIS OF CHROMATIN CONDENSATION BY *HOECHST 33342* STAIN

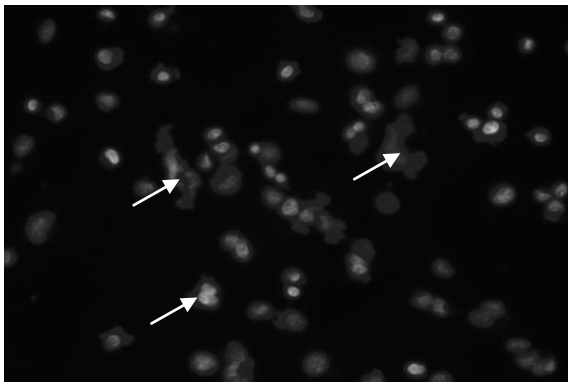
Nuclear chromatin condensation in drug treated MDA-MB-231 cell by Hoechst staining is a visually detectable assay to quantify the amount of apoptotic cells after drug treatment. After treatment with curcumin and EGCG for 0-24hr in a time dependent manner the MDA-MB-231 cells were stained with ***Hoechst 33342 stain*** and analyzed for the chromatin condensation. The result indicates that there was formation of more condensed chromatin structures after treatment with EGCG and curcuminthan control in a time dependent manner.



Control



Curcumin (25µM)



EGCG (200µM)

Fig 12: Observation of Morphological changes in nuclei of treated MDA-MB 231 cells by Hoechst staining

5) COMET ASSAY TO MEASURE THE DNA DAMAGE

MDA MB-231 cells in culture after treatment with curcumin and EGCG for 24hrs were analyzed for the amount of DNA damage by the comet assay. The results showed significant amount of DNA damage in EGCG treated cells and Curcumin treated cells as compared to the untreated control cells. The characteristic comet tail length, tail moment and tail DNA% suggests the amount of DNA damaged which are graphically represented and tabulated below.

Table no. 6: Analysis of comet assay of curcumin and EGCG treated cells

Drug	Drug concentration	Tail length	Tail moment	Tail DNA %
control	0µM	45	5.936982331	13.1932940681554
curcumin	25µM	130.4	21.32449134	16.3531375332227
EGCG	200µM	115.26	27.00348397	23.42832203

From this analyzed data obtained from curcumin and EGCG treated MDA-MB-231 cells by using softwareImageJ, we can plot a graph as shown below:

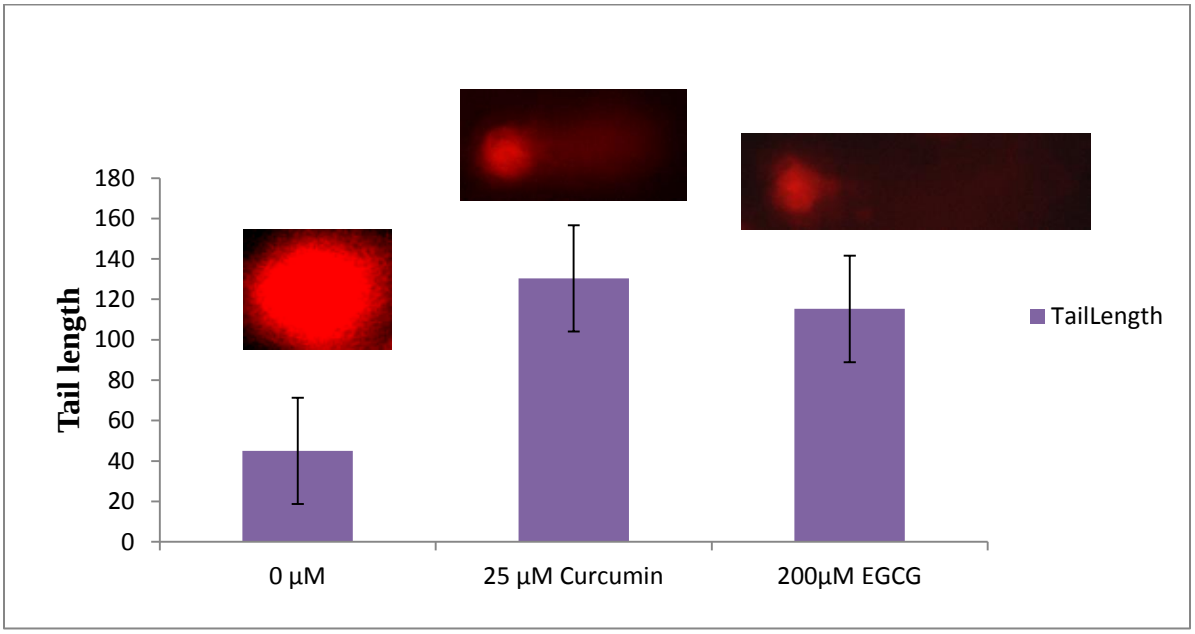


Fig 13: Tail length of curcumin and EGCG treated cells showing DNA damage

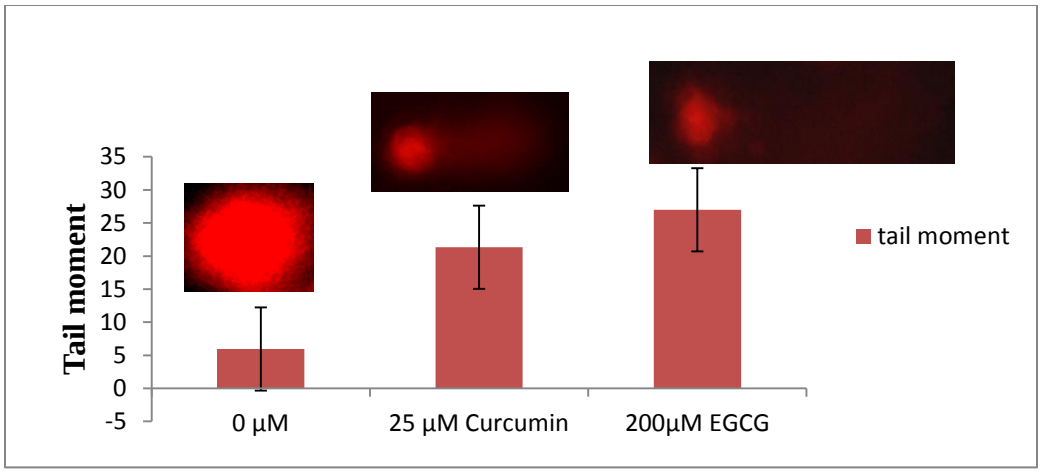


Fig 14: Tail moment of curcumin and EGCG treated cells showing DNA damage

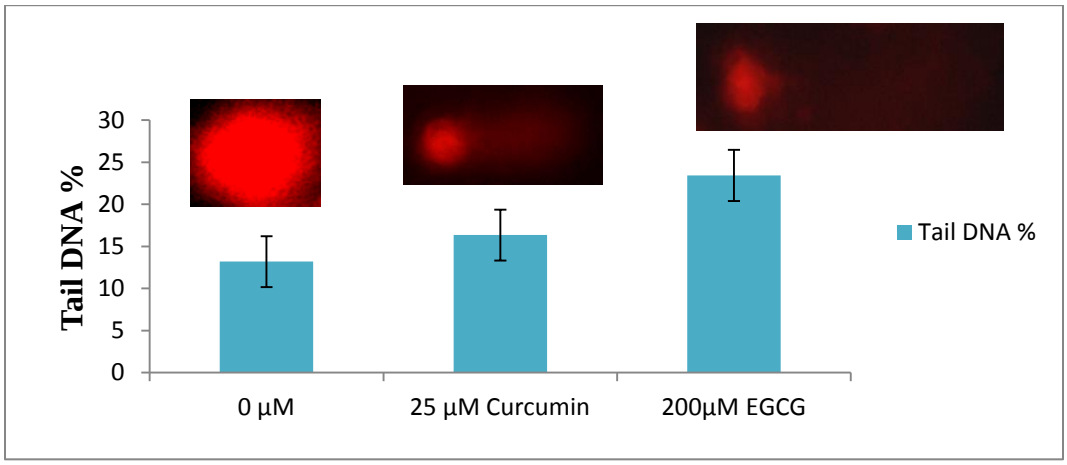


Fig 15: Tail DNA% of curcumin and EGCG treated cells showing DNA damage

6) DNA FRAGMENTATION ASSAY:

DNA fragmentation assay shows that untreated normal cells show a very high molecular weight DNA and remain intact near the lane whereas a ladder of equal size was seen in drug treated cells which show apoptosis. Densitometric analysis of ladder lanes were done using Image-J software which showed distinct peaks representing a single band on the gel, ranging from high to low molecular weight.

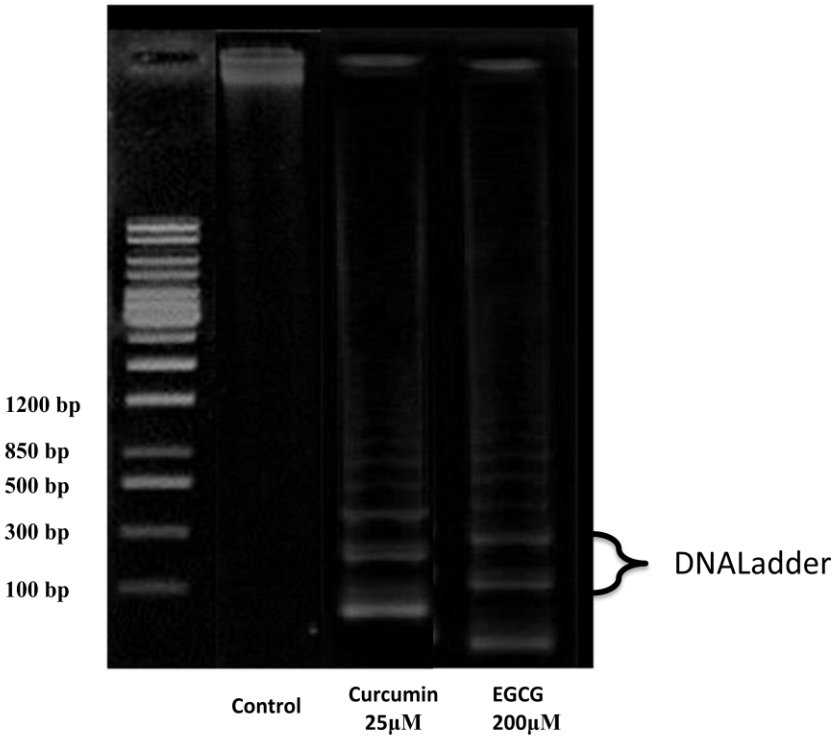


Fig 16: Analysis of apoptosis by DNA fragmentation assay

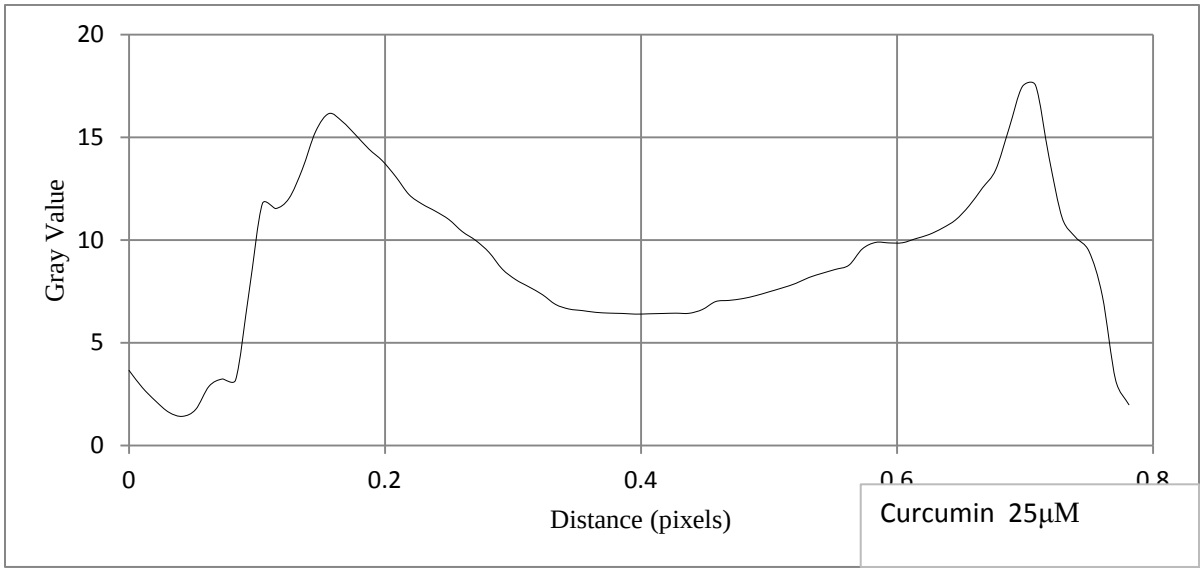


Fig 17: Confirmation of DNA fragmentation in presence of corcumin

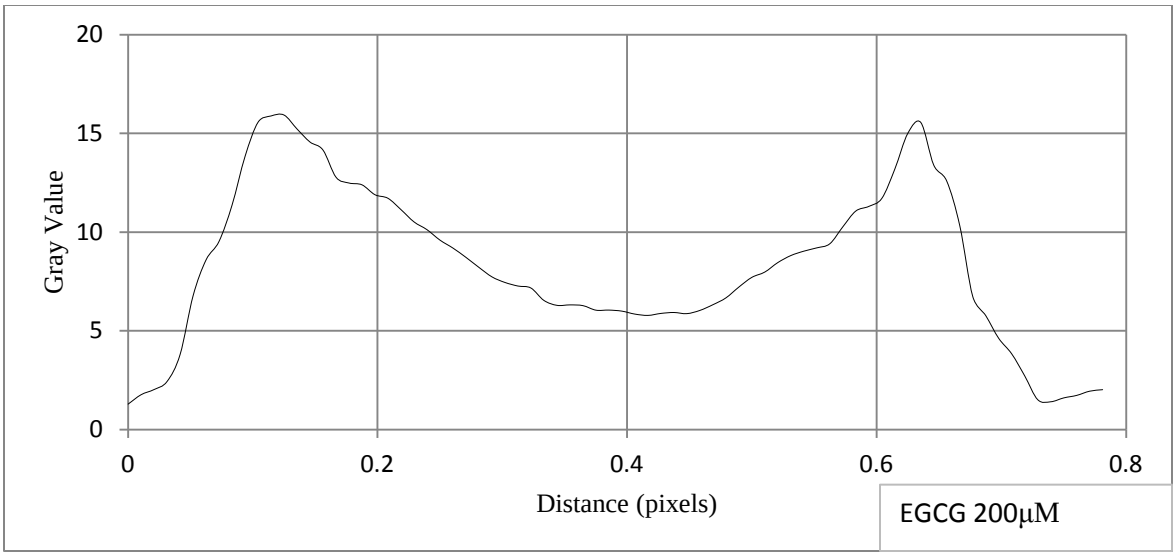


Fig 18: Confirmation of DNA fragmentation in presence of EGCG

7) COLONOGENIC CELL SURVIVAL ASSAY

MDA MB-231 cell's survivability and proliferation was determined by performing colonogenic cell survival assay. This assay determines the reproductive death of cells after treatment of various drugs. From this assay it can be concluded that cell's survivability was declined in the presence of drugs like curcumin and EGCG with respect to control having concentration 25 µM, 200 µMand 0 µM respectively which are shown below in Figure no. 16 and 17.

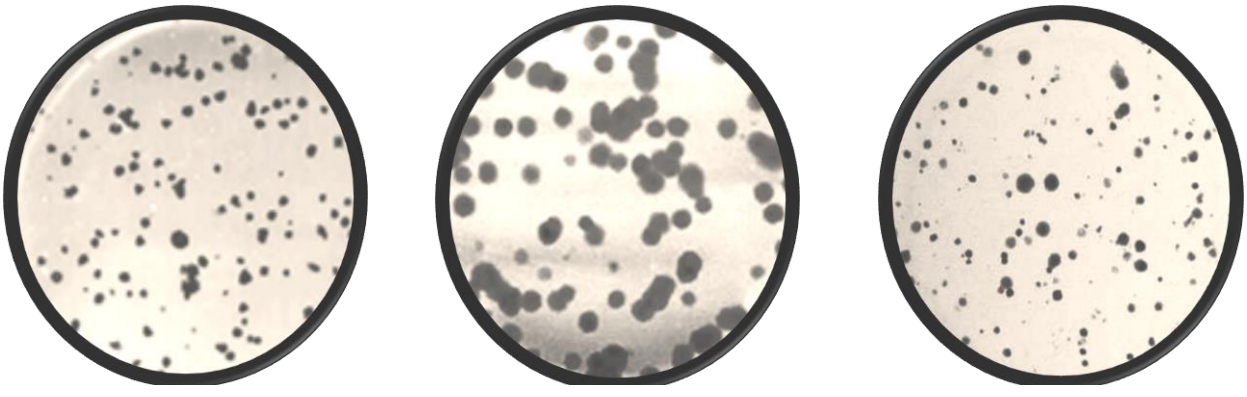


Fig 19: Colonogenic cell survivability of curcumin and EGCG treated MDA-MB-231 cell

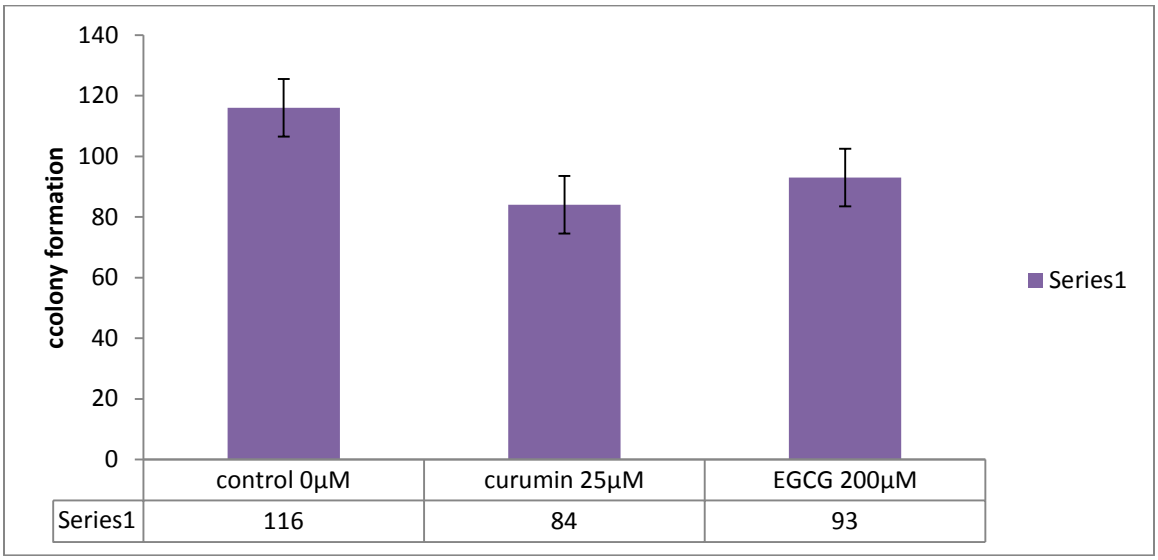


Fig 20: graphical representation of colonogenic cell survivability of curcumin and EGCG treated MDA-MB-231 cell

8) Soft agar assay:

Reduction in the colony formation and anchorage independent growth, is one of the hallmark of cell transformation by colonogenic assay was further analyzed by performing soft agar assay where the cells were grown in semi -solid media which mimics the in vivo condition.Anchorage independent growth is one of the hallmark of cell transformation, was determined by soft agar assay. The proliferation of curcumin and EGCG treated MDA MB-231 cells in a semisolid culture media after 1week was determined by this assay. After 1 week of culture of cells in semisolid media, the cells were analyzed by using software and it was inferred that the survivability and colony formation capability of MDA MB-231 was declined in the presence of curcumin and EGCG as shown in figure no 22 and 23.

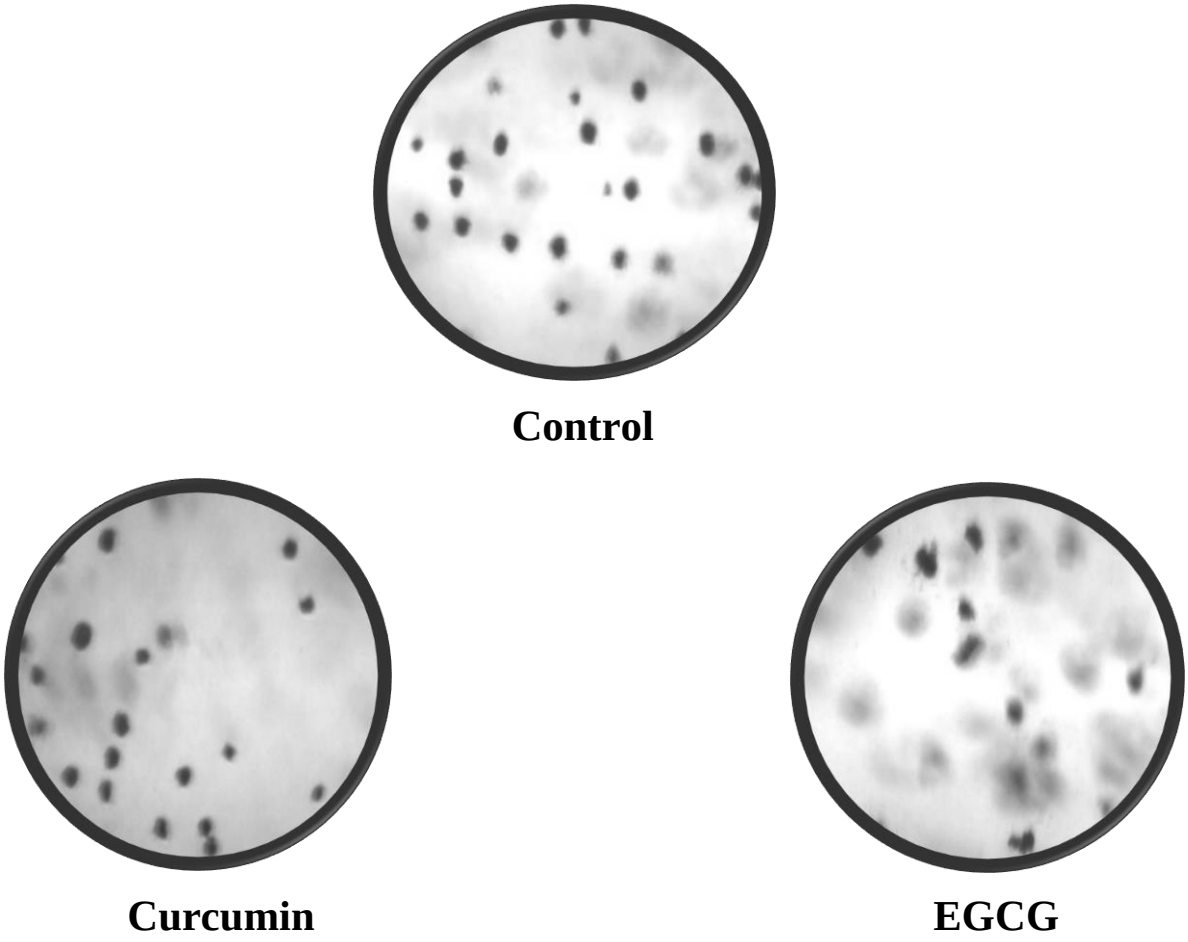


Fig 21: Observation of colony formation in soft agar of curcumin and EGCG treated MDA MB-231 cells

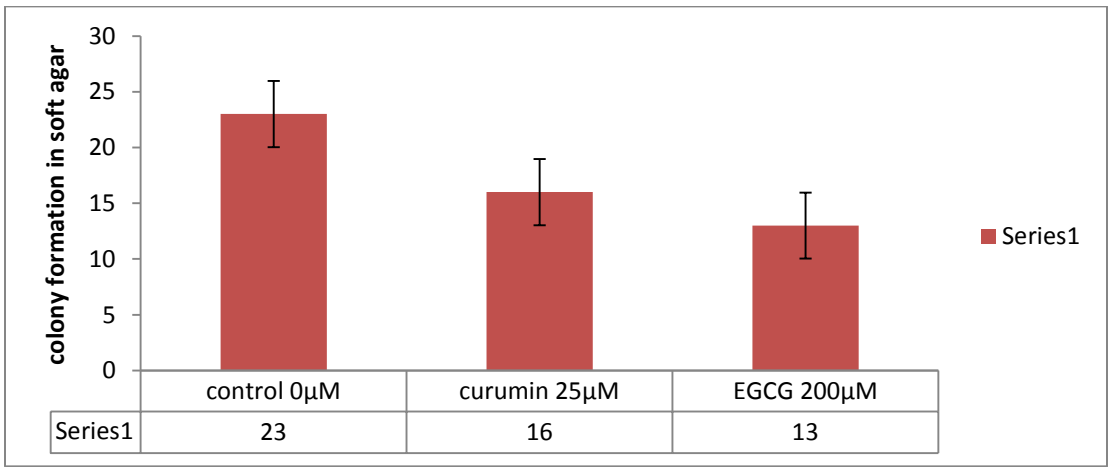


Fig 22: Analysis of Soft agar assay of curcumin and EGCG treated MDA-MB 231 cells

CONCLUSION

Apoptosis is a programmed cell death process, generally characterized by distinct morphological characteristics and energy dependent biochemical processes and considered as an important and vital component of many processes like normal cell turnover and cell death. This process is necessary to eliminate damaged and dangerous cells from the body and to keep body's cell number constant. Inappropriate apoptosis may cause many human conditions including autoimmune disease, many types of cancer etc. PI3k/Akt and RAS/MEK/ERKsignaling cascade are most frequently mutated pathway in human cancer. Their genes are mutated in such a way that they cause deregulation in its effector pathway and various other transcription factors which control cell growth, proliferation, survival and migration. Usually K-Ras and AKT 1 are the most frequently mutated oncogene in human breast cancer. From this study it was observed that the expression level of these two genes were downregulated in breast cancer cell line MDA-MB-231 when treated with two natural polyphenols curcumin and EGCG. MTT assay shows that curcumin and EGCG has more potential to reduce the cell viability but in case of curcumin IC_{50} is obtained at very low concentration about 25µM as compared to EGCG which comes at 200 µM. Also in the presence of curcumin and EGCG this cell line shows more condensed chromatin which is a key hallmark of apoptosis. This result was confirmed by performing comet assay by analyzing the tail length in pixel units. Large tail length was seen in the drug treated cells which is the main characteristic feature of DNA damage. Colonogenic and soft agar assay both can be done toidentify the colony formation capability of cells but soft agar assay has been done in a semi-solid growth media which mimics the *in vivo* condition. From these two assays it was concluded that very few numbers of colonies were formed in these two drugs treated cells, indicating the reduced rate of anchorage independent cell survivability and proliferation but on comparing these two drug treated cells, curcumin has more potential to reduce the colony formation and cell survivability. So it can be inferred that these two natural polyphenols can induce apotosis, inhibit proliferation and downregulates the level of oncogenes at transcriptome level.

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